

15 April 1982

The Falklands from below

Argentina's seizure of the Falkland Islands, a test of honour for Britain that may cost it the Thatcher government, is also a test of President Reagan's peacekeeping abilities.

What is to happen to the great white continent to the south, Antarctica, which has no voters to cast down governments or crowds to wave approving placards in the streets. It may seem trivial to talk about obscure Antarctica, currently a haven for scientists of many nations, its antique sovereignty disputes, in this solemn moment. But the Falklands fiasco has shown how rapidly the obscure far south can be suddenly important. Whether it brings war between Argentina and Britain or a stalemate and negotiations, the dispute has three serious implications for the Antarctic, a region that starts at 60 degrees south latitude, beginning south of the Falklands and South Georgia, the southernmost island taken by Argentina on its weekend spree.

First there is the extreme vulnerability of human settlements in the far south to military takeover. Most of the Antarctic stations south of South America consist of a gaggle of huts nestled against steep black mountains and glacier snouts, the men in bright coloured parkas providing the only colour in a nearly lifeless landscape — except for the thousands of screeching birds — the huts bristle with antennas, but except for a passing ship, the only communication with the outside comes by often poor radio.

The settlements in the Falklands and at Grytviken in South Georgia, different only in that they had real buildings and greener hills, were easily overwhelmed by Argentine forces coming from the mainland a few hundred miles away. It would be harder but not much for a Latin-based military force (or one based in the Falklands) to make the 800 mile crossing of the Drake passage and seize the remaining four British stations there — although such a seizure would be a willful violation of the 1957 Antarctic Treaty that demilitarizes the region, and to which both Britain and Argentina belong. It is unlikely that Argentina would risk the enormous international opprobrium that would follow such a move, but actions in the Falklands remind us how fragile are these sparse southern stations, and how valuable is the Antarctic Treaty.

Indeed, there is a real question as to whether the British Antarctic Survey, which runs the UK's science programme in the Antarctic and until now staged its far-south operations from Stanley and Grytviken, can continue to operate, although it has contingency plans (see page 593). At present, perhaps two dozen British scientists and technical personnel are either being held or are at large on South Georgia — a place whose mountainous hinterland is so impassable that one needs an axe to cut steps in the ice to cross its inland glaciers.

The second implication of the Argentine seizure is oil. According to a report presented in January by Washington energy consultant Lawrence Goldmuntz, the offshore region between Argentina and the Falkland Islands is one of four major "horizons" in the world outside the Middle East capable of producing oil on the scale of the North Sea. Recently, Exxon drilled three wells about midway between the coast and the

Falklands; one was dry, one had gas and one produced 5,000 barrels a day in test drilling. Nearer the Argentine coast, a shell well showed 3,000 barrels a day. So the theory goes, Argentina, beset by economic woes, seized the Falklands to assure supply of a new oil well.

For the most part the oil theory is rubbish. Argentina is very nearly self-sufficient in oil, and by inflaming a dispute to ownership of the Falklands, it would certainly not encourage major oil companies to take out leases. Despite having one of the widest, potentially richest continental shelves in the world, big oil companies have not exactly flocked to do business with Argentina. While its own near-shore shelf and Tierra Del Fuego remain undeveloped, it is hard to justify a push in the unexplored far-off Falklands. Yet the dream of oil wealth may have played a subliminal role in the seizure of the Falklands, and will play a key role in the disposition of Antarctica. Just to the south in the Weddell Sea (claimed by Argentina, Britain and Chile) sedimentary rocks are many kilometres deep while the shelf itself is larger than all of Venezuela. Argentina, West Germany, Norway and even the Soviets have been surveying the geology of the region, to get basic geological data as well as some idea of its oil potential.

The Falklands experience shows that arcane notions of national sovereignty, when combined with the dream of great oil potential, can be an explosive combination. It is easy to laugh at the obscure theories under which seven nations, and potentially several others, claim national sovereignty in Antarctica. It is also easy to dismiss the Antarctic's oil potential as purely hypothetical, as no serious exploration has been done. But the Falklands experience also shows that, nonetheless, the potential for international mischief is great.

The third and most important implication of the Argentine seizure of the Falklands is that it strains international relations at the far south at a delicate moment in Antarctic diplomacy. Argentina itself convened last year the first major international discussion by the 14 Antarctic Treaty powers of how they should dispose of Antarctic minerals — a subject not covered by the original treaty. A second meeting will be held in New Zealand in June, and the list of full parties to the treaty includes many of the key players in the Falkland Islands dispute. After the Buenos Aires meeting, the group showed some promise of being able peacefully to resolve this contentious issue — going as it does, to the heart of the sovereignty dispute. But if Britain and Argentina must use the New Zealand meeting to pound shoes on the table over the Falklands, the result could be disaster for Antarctica.

Clearly, the boundary of the Antarctic Treaty area at 60 degrees south latitude, which in reality is only a windy, stormy sea, should be treated as an iron wall diplomatically by Argentina, Britain and the other Antarctic Treaty nations. Every effort must be made to keep the dispute — no matter where it stands then — from spilling over into the Antarctic question. This will be difficult indeed, but the parties would do well to remember that the Falkland Islands are only 6,200 square miles, about the size of Connecticut or Wales. The Antarctic area, besides comprising an entire continent, one fifth of the world's ocean water by volume, and its largest potential fishery, contains one fifteenth of the Earth's surface. It would be a shame if a dispute over these tiny islands, however serious, were to spoil it.

US contributors please note

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Graduate students galore

The government document on British graduate education fails to grasp conspicuous nettles.

The poor, it is said, are always with us. So too are graduate students, called postgraduate students in the United Kingdom. And the analogy with the poor is not misplaced. One of the strongest threads running through the report of the working party on postgraduate education in Britain set up by the Advisory Board for the Research Councils in 1979 and published last week is the evidence it provides for the impoverishment of graduate students while following their courses and the poor financial prospects which await them afterwards. This is one reason why any thorough study of the arrangements for organizing and supporting postgraduate students must also ask whether the whole enterprise is worthwhile. This study*, which bears the unmistakable stamp of its chairman Sir Peter Swinnerton-Dyer, has done this and more. While concluding that graduate students (a comparatively recent innovation in British universities) have come to stay, the working party has also provided a view of the working of the British system of higher education that could not otherwise have been obtained.

Part of the trouble with graduate education is that it does not fit in easily with the rules that the administrators of higher education have developed for deciding how much money should be spent on supporting universities. So much is evident from the length of time taken after first graduation for different students to qualify for a PhD or some equivalent research degree. Why should some students take only two years, and others five or more? What, in any case, is signified by a PhD degree? Does it denote competence in research, or extra knowledge in some special field or is it merely a sign that its usually proud possessor has qualified by earlier performance for financial support or has been sought after as a research assistant by some supervisor looking for help with his own research? Because circumstances may make each of these readings valid, it is natural that administrators should be at a loss to know how much public support should be devoted to graduate education. These are some of the perplexing questions that seem to have prompted this working-party's study.

So far as they go, the recommendations of the working party are sensible enough. Yes indeed, it says, the time has come for the British government to give up the attempt that its predecessors have made ever since the Second World War to justify spending on university research by means of some calculation that there is a direct link between research expenditure and the improvement of national prosperity. Moreover, the argument goes, it is also impossible, or at least impracticable, to relate public expenditure on the support of graduate students to the needs of employers in different fields. For what employers consider to be their needs are moving targets. The only substantial exception to this chastening truth, the report concludes, is that there may be a continuing need for short-term graduate courses in fields such as computer science or toxicology, where shortages of skilled manpower have recently become apparent — and where they may nevertheless disappear without reason any time. So, the argument goes, the research councils should be quicker off the mark both in their willingness to support courses such as these, if necessary at universities chosen centrally — and then more ready than they are at present to close them down. Nobody will dissent from these conclusions. How readily will the research councils adopt them?

In at least one important respect, however, it is unfortunate that the working party has not gone further. As things are, the chief source of support for graduate students at British universities are the research studentships handed out each year by the research councils. The chief recipients of these grants are not, however, students but the departments which eventually house them and which are, within the rules, then empowered to select the students they consider to be best qualified for a career in research. The working party recommends that the research councils should be more vigilant than in the past in matching the

quotas to the reputations of their dependent departments in research — and that departments whose students seem to take too long to qualify for their degrees should be penalized. The snag is that in present circumstances, the principle of the quota system needs radical re-examination. When the whole pattern of British higher education is being changed in such a way that some university departments will become centres of excellence and others in the same field will have virtually to abandon hope of ever being strong in research, is it equitable or even wise that the appointment of state-subsidized graduate students should be left entirely to the lucky (and no doubt deserving) university departments? Should there not at least be some device for making sure that promising people from less favoured universities have a chance?

The working party might also have profitably gone further in its examination of the character and quality of graduate education in British universities. It seems to have been mesmerized by the publicity given in recent years to the sharp difference between the tenor of PhD courses in the natural and the social sciences. In the natural sciences, it seems to be accepted that in three years or thereabouts, a person can pick up a sense of how to conduct research and also to carry out a substantial piece of work, but that in the social sciences it takes three years to acquire "research training". So, the working party argues, the social science departments of the United Kingdom should tailor their graduate courses to the acquisition of research skills; whether or not successful students are then given a PhD degree becomes irrelevant. But how does it arise that in the United States and elsewhere, the normal pattern of graduate courses in the natural sciences includes a more substantial element of research training ("taught courses" and all that) and often a longer time in harness? Has the committee plumped for three years as the normal period of graduate education leading to a PhD out of deference to the British Government's wish to economize where it can, and without sufficient regard for the quality of graduate education?

The economic value to students of graduate education in British circumstances is exceedingly problematical. Starting salaries for PhD graduates tend to be lower than those of people who find jobs immediately after their first degree, no doubt because substantial proportions of PhD graduates choose to take jobs in academic life. The working party's survey of employment among British graduates of various kinds does not pretend to throw light on economic prospects in the longer term, which is entirely forgivable. But the data that it has uncovered are sufficient to give the lie to the now common supposition in Britain that graduate education is an extravagant luxury, a way in which perpetual students may forsend the harsh realities of the real world for a few years — and often at the public expense. So the working party is right to flirt with the notion that the public stipends of chosen graduate students should be linked not with the grants paid to publicly-supported undergraduates but with those of research assistants doing real jobs in a laboratory environment.

Fine tuning such as this will help to make the present system function more efficiently, but will not by itself revivify graduate education in British universities. Traditionally, PhD courses have been devices for selecting future academics but, when universities are shedding staff, there are many fewer opportunities than aspirants. Is it then surprising that many graduate students become dispirited, and give up along the way? So the most urgent need is that universities should acknowledge that even the most talented graduate students will need to find other than academic jobs. Industrially related graduate courses, such as those sponsored by the Science and Engineering Research Council are valuable but will not suit every need. So should not universities pay more attention to the fate (as well as the selection) of their graduate students? And should not more of them acknowledge that there is a crying need for more than one-year graduate courses designed deliberately to round off the standard three-year first degree course by the acquisition of some employable skill?

*Report of the Working Party on Postgraduate Education, Cmnd. 8537, HMSO, £7.00.

Antarctic research hit by crisis

BAS counts the cost of island invasion

The present dispute between the United Kingdom and Argentina over the Falkland Islands has raised fears about the future of the British Antarctic Survey. But according to the survey's director, Dr Dick Laws, the invasion of the islands by Argentina came too late to have much effect on this season's work, with bases on the Antarctic continent already preparing for the long isolation imposed by the austral winter.

The British Antarctic Survey depends on the Falkland Islands to maintain contact with its research stations. The US National Science Foundation has agreed to provide a temporary commercial relay service via its base on the Antarctic peninsula, Palmer, stressing that it would only pass on messages of a scientific nature and would not handle those dealing with "political matters". In the long term, though, the communications problem could be resolved by setting up a new relay station, probably at the existing British base on Signy Island, in the South Orkneys. The Falklands are also used by the survey as a supply base, and although not indispensable, loss of those facilities would undoubtedly add to the difficulty and cost of operations. Alternative refuelling facilities within easy reach of the British bases are in the Magellan Straits, but so far Chile has not offered use of these.

Direct confrontation between the survey and the Argentinians has so far only occurred on South Georgia, where the commander of Grytviken base is also the senior representative of the Falkland Islands government. Grytviken, base for 26 of the survey's scientists at the time, had earlier been the scene of fighting between Royal Marines and Argentinian forces, although it is not yet known whether the base suffered any damage. Operations at Grytviken were in any case due to be reduced as from this month following budget cuts, despite pressure to maintain a British presence there. South Georgia has recently become a centre for work on krill, whose study as the dominant planktonic organism in the Southern Ocean is important for the exploitation of the Antarctic seas.

These problems come at the end of a troubled season for the British Antarctic Survey. In November the survey lost its two aircraft in hurricane-force winds at Rothera Station, resulting in the cancellation of virtually all Earth science projects, which rely heavily on air support of field parties. Damage to a De Havilland Twin Otter aircraft and the research vessel



South Georgia, showing the British Antarctic Survey's base at Grytviken (left) and the former whaling station (foreground).

John Biscoe the previous year had already meant abandoning that season's marine biological work, and cutting short other research programmes.

However, the future for what is widely acknowledged to be one of the most cost-effective research organizations operating in the Antarctic, is not entirely bleak. The Natural Environment Research Council, who provide the survey's annual funds of £5.6 million, have provided an additional £1.8 million for the purchase of two new aircraft, and £1.3 million for the rebuilding of an important station at Halley Bay, now nearly ten years old. Collaborative research with West Germany is another route that the survey has been exploring to keep within its budget.

The Argentinian action will probably not immediately affect the activities of

other nations in the area, which include the United States, the Soviet Union, Poland, Chile and Argentina itself, as none of these rely upon the Falklands for their communications or supplies. Although Argentina's own research efforts have been extremely limited, it is at present collaborating with the French in glaciological work, and plans exist to site a ground station for the first European Space Agency remote sensing satellite to be put into polar orbit, at the Argentinian base of Marambio.

Clearly the Falklands invasion is going to affect Argentina's standing amongst the Antarctic Treaty nations, due to meet in Hobart in two months' time to discuss the future exploitation of a region with a hitherto exemplary history of peace and international cooperation. **David Millar**

Graduate students on shorter lease

British postgraduates take too long to complete their PhD theses, according to the report of a working party on postgraduate education published last week. Blame is laid primarily on the universities, although the research councils are also criticized for not taking matters more firmly in hand.

The working party, set up in 1979 by the Advisory Board for the Research Councils, found wide discrepancies in PhD submission rates between universities and between the natural and social sciences. Its recommendation that the research councils award postgraduate quotas on the basis of a department's past completion record is being taken up by the Science and Engin-

earing Research Council (SERC) in deciding quotas for 1982-83. SERC has also published a pecking order of universities based on PhD completion success which is intended to shame universities into remedial action.

The working party was set up two years ago under the chairmanship of Sir Peter Swinnerton-Dyer, then vice-chancellor of the University of Cambridge, to advise on postgraduate education policy. It has managed to comply with its remit to assess the ways in which research councils make awards to universities and particular disciplines and whether sufficient postgraduates of the right quality are produced. But the task of determining

whether postgraduate education is meeting manpower needs proved too complicated, chiefly because postgraduates often find employment in areas outside their disciplines of study and employers are usually unable to indicate precisely their future needs at postgraduate level.

The working party report concludes that postgraduate education should continue much at the present level, but suggests that the Natural Environment and Social Science Research Councils should take a leaf out of SERC's book by encouraging specific postgraduate advanced courses in subjects for which there is a growing need.

PhD success rates of SERC-supported students by subject

Subject	Success rate (%)
Biology	68
Chemistry	75
Physics	61
Maths	56
Astronomy	58
Engineering	52
Social science	39
Average	58.4

The data are for 1974 and 1975 starts, and were collected in July 1980–February 1981.

Strangely enough, however, unemployment rates are greater for those who have followed advanced courses than for those who have completed research studentships and, in the same breath, the working party also recommends that SERC should move towards research rather than advanced course studentships. It points to the reluctance to wind down courses once a specific manpower need has been met and SERC's difficulty in finding students of sufficiently high calibre to fill the places reserved for advanced engineering courses.

The universities come in for the worst drubbing. Poor PhD completion rates are attributed mainly to the morale of particular departments and the lack of adequate supervision. Poor supervision, the report says, can lead students to an unwise choice of research topics and can result in inadequate training in the techniques of research.

On the substantial differences in PhD completion rate between the natural and social sciences, the report says that there are inherent differences between the subjects. Thus social science students concerned that their first work should be a *magnum opus*, have a greater tendency to take on research topics which are far too ambitious to be completed within the normal three years. Perhaps, therefore, it is no surprise that 54 per cent of those embarking on PhDs in the natural sciences in 1975 had completed within five years compared with only 31 per cent in the social sciences.

Nevertheless, the working party finds the PhD completion rates in both disciplines "wholly unsatisfactory". The criteria for a PhD may have become more stringent in recent years, says the report, leading some students to take up topics unsuitable for a three-year study. Another

Average salary of graduates by occupation and highest postgraduate qualification achieved (1979–80)

<i>Engineering and technology</i>	
None	£5,520
MA	£5,700
PhD	£5,085
<i>Science</i>	
None	£5,350
MA	£5,970
PhD	£4,855
<i>Social studies</i>	
None	£5,810
MA	£5,970
PhD	£5,000

problem is that students from small departments can lose motivation, especially if inadequately supervised. It therefore recommends that the research councils concentrate their postgraduate awards in larger departments where students may be attached to groups of experienced researchers working on a particular problem, and that supervisors should take more care to fulfil their responsibilities. The aim should be to complete research work within three years and to submit a final thesis before the end of the fourth.

The working party's report recommends several remedial actions. Universities should monitor research students at the end of their first year of postgraduate study and relegate those unsuited for research to MSc courses. Both outside academic institutions and industry should have a greater role in the choice of an individual's research topic. Finally, the research councils should review their postgraduate policies every four years, taking into account representations from potential postgraduate employers. **Judy Redfearn**

University research staff

Postdocs' plight

More than a quarter of the academic staff at the University of Bristol are contract researchers — postdoctoral students who in years gone by would now be on the academic staff. This is one of the more startling conclusions of a detailed survey of Bristol academics just completed by the lecturers' trade union the Association of University Teachers (AUT).

AUT claims that this is the most comprehensive study of university employment yet undertaken in Britain, and it reveals the frightening extent to which universities appear to be relying on labour which, if the research councils firmly apply their rules of cutting off postdoctoral support after, say, six years, could rapidly be lost. Nearly two-thirds of the contract researchers have less than a year of their present contract to run, the survey showed. Thus the university system could shrink without the government lifting a finger.

Naturally, AUT is most concerned about the individuals who may lose their jobs. A third are over 30 years old, one in five has dependent children, and nearly 40 per cent of researchers who have either masters or PhD degrees have no "academic status" (granting them voting and library rights, for example) in the university.

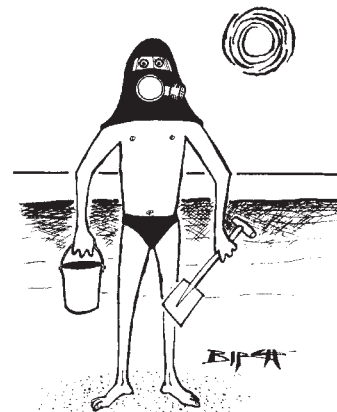
The survey, says AUT, "shatters the myth" that contract research can be regarded as a brief step on the way to an academic post. Nine per cent of the researchers surveyed had had four or more contracts, and 34 per cent, two or three. Twenty-eight per cent of the contract researchers had already been in research

Med comes clean

Political bickering between the 18 countries surrounding the Mediterranean has not stopped the initialling of the fourth protocol and arm of the United Nations Environment Programme's (UNEP) plan to save the Mediterranean from further environmental damage. The Mediterranean action plan was launched at the Barcelona convention in 1976, and the latest agreement obliges the signatory countries to set up coastal protected zones. The aim is not just to preserve a representative cross-section of Mediterranean ecosystems but also to foster commercially viable farms for oysters, lobsters and other shell fish.

The 1982–84 budget of \$7 million will mostly be shouldered by Italy, France and Spain, with \$400,000 from the EEC itself. A major spur for this spending is the concern that environmental dangers will affect the tourist trade. From a scientific point of view the protected areas will be useful for baseline studies of ecosystems to see whether coastal waters are getting worse or better.

Among the Mediterranean species in danger of extinction are the monk seal, marine turtle, European otter, Dalmatian pelican, peregrine falcon, oysters, mussels, the Iberian midwife



toad, the pied kingfisher and the spectacled salamander. UNEP hopes that the present number of 15 marine parks and reserves will eventually grow to at least 100. **Jasper Becker**

In a report on the survey addressed to the university administration, AUT makes thirteen recommendations, most of which entail effectively putting contract workers on the staff.

According to AUT, the contract researchers should be given academic status, salary scales and a career structure. The university should have a contingency fund to support contract researchers between contracts, and it should "exert pressure" on the research councils to develop "a more positive approach" to the employment conditions associated with their contracts (which support 60 per cent of the researchers concerned). AUT also recommended that the universities should abolish waivers in contracts, which absolve them from paying redundancy pay and remove the right of appeal against unfair dismissal.

● In a survey of the employment prospects for postgraduate astronomers in Britain, the Royal Astronomical Society (RAS) has come to conclusions which broadly confirm the AUT study at Bristol (see above). Some 42 per cent of respondents were in the fourth or later year of a postdoctoral appointment. And 40 per cent of non-tenured astronomers are over the age of 30. RAS respondents were also asked if their degrees and research enhanced their employment prospects. The proportion saying yes fell with increasing qualification, from 79 per cent for the first degree to only 56 per cent for postdoctoral research.

Robert Walgate

British Aerospace

Looking good

A new mood of optimism prevails at the space and communications division of British Aerospace. Not surprisingly, the company is delighted at last month's news that the Hughes Aircraft company is to build up to 16 Intelsat-VI telecommunications satellites: as principal sub-contractor British Aerospace stands to win orders worth up to £50 million over the next seven years. And having also won the contract to build the European Space Agency's next generation telecommunications satellite, L-sat, the company is now said to be working to capacity.

The arrangement with Hughes over Intelsat VI, however, is just one step towards what is hoped will develop into wider cooperation. British Aerospace, through its participation in the programmes of the European Space Agency, believes that its credibility is now well established in Europe but that it will never enter the US market alone. Thus the link with Hughes is cherished.

The Intelsat VI contract could be worth \$1,600 million to Hughes if options on all 16 spacecraft are taken. The plan is to build five spacecraft initially for launch aboard Ariane or the space shuttle from 1986 onwards. Hughes has taken many of the prizes in the Intelsat developments — only

contracts for Intelsat III and V were awarded to other companies, TRW and Ford Aerospace.

The capital for the Intelsat VI development will be raised by the 106 member organizations which consist mainly of national telecommunications services. The largest shareholders are the United States, with 20 per cent of the shares, and British Telecom which holds a 13 per cent share. Intelsat VI will carry 33,000 voice and four television channels, more than twice the capacity of Intelsat V. The design, which incorporates a satellite-switched time division multiple access capability for digital transmission, may be up-graded to 100,000 voice channels later.

Judy Redfearn

UK cancer research

Marking time

Britain's leading cancer research laboratory, the charitably supported Imperial Cancer Research Fund (ICRF), claims it is running short of cash — despite last year's income being the largest ever. New fund-raising mechanisms are being sought, including high-street shops and — in the long term — biotechnology.

Where has all the money gone? ICRF was criticized a few years ago for being overcautious, salting away charitable pennies in an endowment fund while spending too little on current research (only 32 per cent of its income in 1971). Now ICRF claims the balance has gone too far the other way: 58 per cent (£10 million out of £17 million raised) was spent on research in 1981, and savings have fallen correspondingly from 44 per cent in 1981 to 20 per cent last year. This year there is talk at ICRF of cuts: perhaps a loss of six staff, whereas over the past three years staffing levels have risen by 50 per cent.

The recent expansion has been the work of Dr Walter Bodmer, present research director, and his deputy Dr Michael Crumpton, but the spending seems to have overshot the mark. Government spending cuts are blamed. Not that the government supports ICRF, but cuts in the Health Service and in universities have meant that research councils, and bodies such as ICRF, have found themselves spending more than expected to provide basic services when they collaborate with hospital clinics or university laboratories.

ICRF commitments to support a new cyclotron for neutron therapy at Clatterbridge Hospital near Liverpool (the ICRF contribution is £3 million), and the building of a new £12-million laboratory site at South Mimms in north London, to replace the Mill Hill site (whose lease runs out in a few years' time), have also eaten into the budget.

The remedy will be to halt the expansion of the research budget and to attempt to raise more money by appeal — difficult in the present economic climate — and by

other means. One plan is to open shops selling second-hand clothes and bric-a-brac along the lines of the operation run by Oxfam, the famine relief organization.

ICRF is also making its entry into biotechnology. The prime objective is not to make money, says Dr Bodmer, although money would clearly be welcome if it came along. Rather, the principle will be to make commercial agreements which will speed the distribution of clinically or experimentally useful products (such as monoclonal antibodies) but to avoid "inappropriate" exploitation of the products outside ICRF control. The fund has engaged patent and commercial agents to help in this activity.

To speed this work, the South Mimms site (which should have opened in 1986) will have what is described as a "pilot plant" for large-scale cultures, which will have the double function of supplying the ICRF laboratories with materials and of testing cultures for potential industrial scale-up.

In the rest of its space, South Mimms will house the research services presently at Mill Hill and at the main ICRF laboratory in Lincoln's Inn Fields. After all the shuffling, space would be left at Lincoln's Inn Fields in 1985–86 for perhaps two new groups. Oncogenes and growth factors are among the research areas that are under consideration.

Robert Walgate

Biotechnology

Endorphin now

University College London last week announced a new joint venture in biotechnology that is more significant as a sign of the times than for the sum of money involved. Nevertheless, for Professor Brian Rabin and Dr Peter Butterworth of the college's biochemistry department, the \$240,000 contract to clone pancreatic endorphin is a godsend when the British government's economies in higher education are beginning to bite.

The contract with University College comes from Endorphin Inc., a company founded in Seattle in February. The company is equipped with venture capital, a patent and a president, Professor John Houck, who uses the money he has raised to support his own laboratories at the Virginia Mason Research Center in Seattle and now to support the University College project.

Professor Houck founded the company on the basis of his discovery that pancreatic tissue contains a hormone of potential therapeutic value. The hormone, not yet completely characterized, is related to the endorphins which also have therapeutic potential, particularly as analgesics. According to Professor Houck, however, whereas intravenously administered endorphins cannot readily reach the brain in an active form, the pancreatic endorphin can do so.

To prove the point and to prepare the way for clinical trials, Endorphin Inc. now needs a quantity of pancreatic endorphin that would be difficult to purify from pig pancreas but should be well within the production capacity of bacteria into which the relevant gene has been cloned.

The choice of University College for this task seems in large part to have been based on the fact that Professor Houck has been a visiting fellow there for several years. It may also have been cheaper to contract for the work in the United Kingdom than in the United States. But for the department of biochemistry, whose annual support from the university for overheads has been cut from £80,000 to £60,000 (an even more drastic cut in real terms), the contract allows at least one project to be carried out

in style.

Dr Butterworth claims that the normal funds available to university departments are now simply insufficient to buy the radioisotopes and restriction enzymes necessary for most gene cloning projects. Nor is there a wealth of British venture capital waiting to help out. Since matters are likely to get worse rather than better he and Professor Rabin hope that the contract from Endorphin Inc. will be followed by others.

They hope also that the gene cloning for Endorphin Inc. is successful enough for the contract to be renewed in two years' time and that pancreatic endorphin eventually reaches the market. If it does University College stands to gain a one per cent royalty on all sales. **Peter Newmark**

Europe leads on sequences

Europe appears, for a change, to have beaten the United States to the mark. The European Molecular Biology Laboratory (EMBL) at Heidelberg has announced the formation of a nucleotide sequence library, while the National Institutes of Health (NIH) in Washington are still deliberating the question.

Not that there is any sense of competition. Greg Hamm, manager of the Heidelberg library, is "still talking" to NIH, and wishes to cooperate with any system that NIH may set up. But EMBL was under pressure from European scientists to start now, before sequence data were irretrievably lost. EMBL is not attempting to become the sole manager of world sequence data, Hamm insists.

The question now is how to collect the data efficiently. The 600,000 nucleotides already logged at Heidelberg (they are freely available on magnetic tape) have taken an immense effort to collect. This is largely because journals do not use the clearest of systems for displaying the sequences, say Hamm and Professor Ken Murray in a letter recently sent to journal editors.

Hamm and Murray are recommending that journals should insist on the separate

submission of sequence data to the EMBL library, preferably in a format specified by EMBL, and if possible in computer-readable form. Some of these requirements, however, conflict with common format in journals. For example, codons should be presented in tens or fifteens of nucleotides rather than the triplets which correspond to the translation of the code into amino acids, says EMBL, to reduce counting errors; and the marking of reading frames and alignment of comparable sequences should be avoided.

According to Hamm, however, such requirements are only important for the easy transferral of the data into the EMBL computer: the printed format in a journal, which is intended to present the data heuristically, emphasizing the significance of regions of a sequence, could be quite different — provided EMBL were sent the uncluttered sequence according to their own guidelines.

So far, only the *Journal of Molecular Biology*, edited by Sir John Kendrew, has said it will implement the EMBL proposals in full. *Nucleic Acids Research* is said to be "basically positive", but discussing details. *Nature* is considering the matter.

Robert Walgate

EMBL

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DT 82.01.01 (first entry)
XX
DE First two exons in immunoglobulin light chain genes from
DE cell line MOPC41.
XX
KW differentiated gene; immunoglobulin.
XX
CC Mus musculus (house mouse, souris domestique, Hausmaus)
OC Eukaryota; Metazoa; Chordata; Vertebrata; Tetrapoda;
OC Mammalia; Eutheria; Rodentia.
XX
RN [1] (bases 1-350)
RA Altenburger W., Steinmetz W., Zachau H.G.;
RT "Functional and non-functional joining in immunoglobulin light
RT chain genes of a mouse myeloma";
RL Nature 287:603-607(1980).
XX
FT Key From To Description
FT
FT CDS 126 176 first exon (leader peptide)
FT CDS 303 >350 second exon (variable part)
XX
SQ Sequence 350bp: 80 A; 82 C; 122 T; 66 G.
CGTGACCAAT CTAATAGCTG TCCTAATAAT TTGCATACCC TCACTGCATC GCCTTGGGGA
CTCTTTATP TAACAGCTCA ACATATCTCTG TGGCATTGTC ATTGCAGTCA GGAAGTCAAG
TGGACATGAG GCGCTCTGCA CAGATTTTGT GCTCTTGTGT GCTCTTGTGT CAAGGTTAAA
ATGAAACTTA AAATTGGGAA TTTCACAGT TTTCACAGT TGGTTAGTGT TCACTGGCAT
TTGGGGGATG TCCTCTTTTA TCATGCTTAT CTATGTGGAT ATTCATTATG TCTCACTCC
TAGGTACAG ATGTGACATC CAGATGACCC AGTCTCCATC CTCCTTATCT
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A sample entry from the EMBL sequence data library, specifying the source of the DNA as closely as possible, giving a reference and listing special features of the sequence in a table (in this case showing the exons). The original is approximately 6 inches wide.

Polish universities

Trial by proxy

New York

The International Council for the Future of the University (ICFU), a New York based organization of some 300 academics from 21 countries, committed to the defence of Western university traditions, has launched a study of the current situation in Polish universities. This marks a major change in ICFU policy. Previous studies have dealt with Western European countries (Sweden, Italy, West Germany) to which it was possible to send working parties and in which the ICFU already possessed members who could contribute first-hand knowledge of recent events. But with no Polish members of ICFU, and with the continuation of martial law the possibility of an ICFU commission visiting Poland in the near future seems remote.

The inaugural meeting for the new study, which was held in New York earlier this month, had to do the best it could with the testimonies of visitors, including Andrzej Kaminski, a mediaeval historian who left Poland before the foundation of Solidarity, two representatives of the banned Independent Students' Union (NZS) and Wojciech Karpinski, a political scientist and former activist now at Yale. Inevitably, their accounts of Polish university history did not cover the period of martial law. The most up-to-date material, including the martial law regulations governing the universities (see *Nature* 31 January, p.181), came from the floor. A detailed discussion of the events which followed the military take-over was deferred to a meeting to be held in Paris later this year.

More incongruously, the reforms urged and in part initiated in Polish universities during the 16 months of "renewal" (between 30 August 1980 and 12 December 1981), had been largely directed towards greater democracy ranging from student representation on the academic councils of universities to the possible election of a Minister of Higher Education by the university rectors — a proposal put forward only a few days before the imposition of martial law, and one which was later described by Deputy Prime Minister Mieczyslaw Rakowski as "the academics running amok". This trend contrasts sharply with the attitude of ICFU, which has tended to see too much democracy as a potential threat. But this divergence of views may make it easier for the Polish authorities to accept the impartiality of the proposed ICFU study. If the Polish propagandists look at the track record of ICFU it might find it difficult to make out a plausible case that ICFU is manipulated by Solidarity extremists abroad, when for the past ten years it has systematically deplored many of the reforms since advocated by Solidarity. **Vera Rich**

Data banks

Right to privacy

Brussels

Following the adoption of the European Parliament's resolution on the rights of the individual with respect to data processing, it now seems that the European Commission may well start work on drafting legislation to protect the confidentiality of information held in data banks. The European Parliament's resolution at the plenary session in Strasbourg last month has particular relevance for the United Kingdom which has so far shied away from tackling the controversial issues of the freedom or protection of information.

Respect for the individual's privacy has become a matter for international organizations like the EEC and the Council of Europe because of the increasing amount of information which is passed across national borders for processing or storage. The fear is that countries without satisfactory legislation on the subject might become information "havens", analogous to offshore banking centres, remote from the judicial control of the country of origin. The lack of adequate laws in the United Kingdom has become a significant impediment to the growth of the UK data processing industry, causing Sweden not to send data to the United Kingdom for processing.

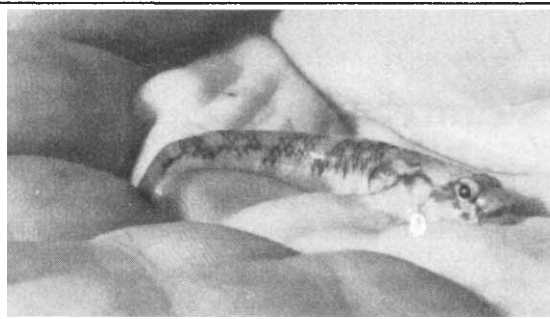
At the moment there are non-binding guidelines drawn up by the Organization for Economic Development and Cooperation (OECD), and a convention for the protection of individuals with regard to automated data files has been approved but not yet ratified by the Council of Europe. The convention sets out the basic principles of data protection. These include the fair and lawful collection of data, restrictions on the storing of particular data and prohibitions on the storing of sensitive data such as political opinions. The convention also gives anybody about whom data are held the right to obtain information about the nature of the data from the agency officially responsible and to demand that inaccurate data are corrected. What categories of data can be excluded from the scope of the convention and whether data-protection rights are given to groups of people, associations or companies are left to the discretion of the signatory countries.

The OECD guidelines, which the United Kingdom, Canada and Australia have objected to, are more in line with the views of the United States, particularly the insistence that data should flow across borders unhindered by administrative checks or delays.

Neither of these international codes is yet in operation although many European countries have well established laws on information and data control. The European Parliament's rapporteur, Hellmut Sieglerschmidt (German, Socialist),

Fishy proof

This painted goby, the one-hundredth species of fish to be found in the River Thames, has been greeted by Thames Water's chairman Geoffrey Edwards as proof that the Thames is the cleanest metropolitan estuary in the world. This follows the "clean-up" campaign of London's sewage system that began in 1960.



concluded that the different national rules are damaging for both the individual and for the data processing industry. In his view, the convention proposed by the Council of Europe leaves too many aspects to the discretion of signatory states. Sieglerschmidt called for a more radical EEC directive. Ideas in this proposal include: the same level of protection in both private and public sectors; the obligation for data holders to notify persons on whom information is held and permit corrections to be made; and the right of the subject of the data to claim for damages against data holders. These proposals were toned down by amendments from Conservatives. **Jasper Becker**

Science parks

Easy answer?

With cuts in government support hitting UK universities hard, the scramble to set up science parks is on — 15 were launched last year. Few university schemes are actually in operation, and newcomers must look to Trinity College, Cambridge, the first British academic institution to open a science park, for the blueprint.

"Science park" is a vogue term applied to many different schemes. Local authority backed projects, such as that in Warrington, aim to attract high technology industry in the hope of creating more jobs. The universities hope that science parks will earn extra income and provide an environment in which academic scientists and entrepreneurs can put new technology onto the market.

In 1973 Trinity earmarked 140 acres on the outskirts of the town for development. Phase I is now complete and the site houses 23 companies. When Napp Laboratories Ltd, a pharmaceutical company, moves into the new building it is constructing, the number of people working on the site will total 1,000. Trinity is well pleased with its park, collecting around £250,000 a year from lease arrangements, and content at having no equity shares in any of the companies. No companies have moved out and several have expanded their premises. "Every major university should have one", commented John Bradfield, bursar of Trinity.

The park's tenants range from small companies founded by one or two business-minded science graduates to

divisions of multinational companies. LKB Biochrom Ltd, the British subsidiary of a Swedish firm, was attracted to the park because of its proximity to a prestigious university. LKB moved to the park in 1974 and produces analytical instruments used in life science laboratories. According to Mr David Storey, LKB's managing director, the attitude of academic researchers to commerce has undergone "a remarkable change over the past ten years". Many members of the university now act as consultants on either an informal or formal basis, in sharp contrast with the hostility met by IBM twenty years ago when it tried to find premises in Cambridge.

Laser-Scan Laboratories Ltd, which came to the park in 1973, grew directly out of a university department. Founded in 1969 by three researchers from the Cavendish Physics Laboratory, Laser-Scan produces machines, used by the Ordnance Survey and the Ministry of Defence, based on laser scanning technology. Products made by the university engineering department's Microcircuits Group, which is supported by GEC, British Telecom and the Science and Engineering Research Council, will be marketed by a company on the park, Lintech Instruments. Although the park has demonstrated, as one research worker put it, that academics need not "wholly prostitute themselves to become entrepreneurs", it is not yet looked on as part of the university. Its distance from the university prohibits such an intimate involvement.

Cambridge Science Park is an example of a scheme established in response to government pleas in the late 1960s that industry and the universities form closer links. In 1969 a Cambridge committee chaired by Sir Nevill Mott recommended expansion of science-based industry close to the university. Dr Bradfield, who has guided the scheme from its inception, sees science parks as a means of educating academics in the commercial development of their ideas. The Cambridge Science Park has, in this respect, proved successful. But to look to science parks for a significant boost for British high technology, or as a way for universities to replace lost income, is optimistic. High technology companies are notoriously high risk ventures and present conditions are not conducive to overnight success.

Jane Wynn

CORRESPONDENCE

US cancer trends

SIR — Your article on the US Surgeon General's report *The Health Consequences of Smoking: Cancer 1982* (*Nature* 4 March, p.4) again draws attention to the importance of smoking as a cause of cancer. The "remarkable increases in mortality from smoking-related cancers", a widely recognized phenomenon, is usefully reiterated by the Surgeon General. The concurrent decline in the death rate from all non-smoking-related cancers, also undisputed, is reported in the *Nature* news item. We would like to point out a misinterpretation of the reasons for the decline in cancers not related to smoking which has followed publication of the report.

The Surgeon General cites "improvements in survival rates for some cancers through earlier or better diagnosis and treatment" during the period of steeply increasing smoking-related cancer mortality rates. As had been well publicized, improvements have been made in the treatment of Hodgkin's disease, some childhood leukaemias, testicular cancer, and a few others. In contrast, however, improvements in survival of people afflicted with solid tumours, those responsible for the vast majority of cancer deaths, have been small. The improvements in survival, while important, have had little effect on overall mortality from non-smoking-related cancers.

The major contributions to reduced non-respiratory cancer mortality are the falling mortality rates for stomach and uterine cancers. These declines result not from improved survival, but from declining incidence rates for these cancers¹.

Stomach cancer rates are now decreasing throughout the developed world. In the United States the trend continues downwards throughout middle age, suggesting that as younger people age, they will have rates lower than those of their parents' generation. Stomach cancer mortality rates in the United States are now among the lowest in the world. Early detection and improved treatment has had little impact on the mortality rates. There has been practically no improvement in survival of stomach cancer patients since 1950.

No single explanation adequately explains the decrease in stomach cancer incidence, but several factors have been suggested as contributors: modern techniques of food preparation and storage, increased consumption of green vegetables, fruits, antioxidants (as food preservatives) and increased milk intake.

The long downward trend in cervical cancer, which began at least 40 years ago, is the chief reason for the large, steady decrease in female non-respiratory cancer death rates over the past half century. The decline began long before the "Pap test" for early detection of cancer of the cervix became widespread. The causes of this substantial improvement are not fully understood, though it appears to have accompanied general improvements in public health and living standards.

The *Nature* news article cites improvements in survival of prostatic, colorectal and breast cancer patients as contributing to reductions in non-smoking-related cancer mortality. In fact, mortality rates for these three cancers have remained nearly constant since 1950.

While the contemporary declines in

mortality from stomach and uterine cancers appear to be fortuitous concomitants of modern life, additional major reductions in cancer rates can be achieved with present knowledge. The evidence indicting smoking provides the greatest unrealized potential for reducing the impact of cancer.

MICHAEL GOUGH
HELLEN GELBAND

Office of Technology Assessment,
US Congress,
Washington D.C., USA

1. *Assessment of Technologies for Determining Cancer Risks from the Environment* (Office of Technology Assessment, US Congress, 1981).

Anthrax island

SIR — We would like to deal with a number of points raised by Dr Sterne (*Nature* 4 February, p.362) regarding our paper on anthrax contamination on Gruinard Island¹.

The main object of the 1979 survey of Gruinard Island was to determine whether or not the whole island was grossly contaminated with *Bacillus anthracis*. We have shown that it is not, but that the organism is found in detectable numbers in a small area.

We believe that Dr Sterne has misunderstood our view about Van Ness's assumption that *B. anthracis* proliferates in the soil under certain conditions². We stated in our paper that we did not believe such conditions existed on Gruinard Island. The present extent of the contamination was probably due solely to the wartime trials with some slight redistribution of the spores by wind and rain.

We agree with Dr Sterne's statement that he failed to recover *B. anthracis* from cadavers of cattle buried as little as three months previously because it is known that only spores survive for any length of time. The vegetative bacilli only sporulate in the presence of oxygen^{3,4}, and veterinary officers dealing with suspected cases of anthrax do not normally open the body cavity thus ensuring that oxygen is excluded.

We have shown that if blood is added to contaminated soil from Gruinard Island the spores will germinate and proliferate. The numbers of indigenous soil bacteria did increase as a result of this procedure but the selective medium allowed us to obtain accurate counts of *B. anthracis* colonies. Dr Sterne rightly states that there is no experimental evidence that *B. anthracis* does multiply in the soil but what we have tried to show is that the possibility of proliferation in the presence of nutrients does exist and should not be entirely disregarded.

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1. Manchee, R.J., Broster, M.G., Melling, J., Henstridge, R.M. & Stagg, A.J. *Nature* 294, 254-255 (1981).
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Improbable stuff

SIR — That the information content of terrestrial biology is truly prodigious¹ seems generally agreed. Hoyle and Wickramasinghe² avoid the problem of assessing repetitiveness and irrelevancy in the DNA (*Nature* 1 October 1981, p.333) by starting from the fact that 2,000 enzymes are common to all life. Each enzyme has a distinct "backbone" of, say, a dozen amino acids together with a specific active site which catalyses a particular biochemical reaction and is determined by, say, six further amino acids. It is presumed that these common enzymes determine a minimum information content in biological DNA.

Taking the twenty biological amino acids, one can compute the number of different combinations of Boltzmann's³ "permutability" P of enzyme-like chains: for 2,000 18-link chains P would be $\sim 10^{40,000}$, for 20,000 18-link chains containing the 2,000 specific ones it would be $\sim 10^{26,000}$, while for 2,000 6-link chains it would be $\sim 10^{13,000}$. In choosing the number of 6 to characterize the active site, Rout (*Nature* 18 March 1982, p.192) discounted the "backbone" but more importantly forgot that activities such as energy release from sugars or accurate DNA replication require sequences of particular enzymes catalysing reactions in a specific order.

Weighting the amino acids according to some expected abundances modifies the numbers but an index of the order of 10,000 is inescapable — that is, a probability of $1:10^{10,000}$ against finding the present enzyme system in a particular sample at a particular time as a chance arrangement. To demonstrate the immensity of such numbers, note that the product of the number of bacterium-sized drops (10^{-13} cm^3) in the upper 1 metre of the Earth's surface water and the number of milliseconds in 4 billion years is 10^{54} . Alternatively, the number of UV photons over 4 eV (capable of breaking and rearranging a chain) arriving through the Earth's history is 10^{49} . All Earth-like planets in the Galaxy throughout its lifetime would give "only" 10^{78} or 10^{73} .

It is probable that some simple self-replicating systems exist that are viable in restricted environments, if only because present biology can prosper in extreme conditions and utilize a wide variety of exothermic chemical transformations². So life might have started from a very simple biology, arising by chance on the primitive Earth. Experimental demonstration of a practical system is not inconceivably remote⁴.

But why don't geocentric evolutionists (*Nature* 18 March 1982, p.198) face up to the awkward questions: where are all the simpler intermediate biologies — surely some should have had advantages over our unnecessarily rich biology and have left clear traces? And why should bacteria be adaptive to non-terrestrial environments and in particular be capable of space travel?

MAX WALLIS

University College Cardiff, UK

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CHARLES DARWIN 1809-1882

The century since Darwin

Charles Darwin was born on 12 February 1809 and died on 19 April 1882. Here John Maynard-Smith reflects upon the 100 years since Darwin's death. A contemporary controversy is discussed in News and Views.

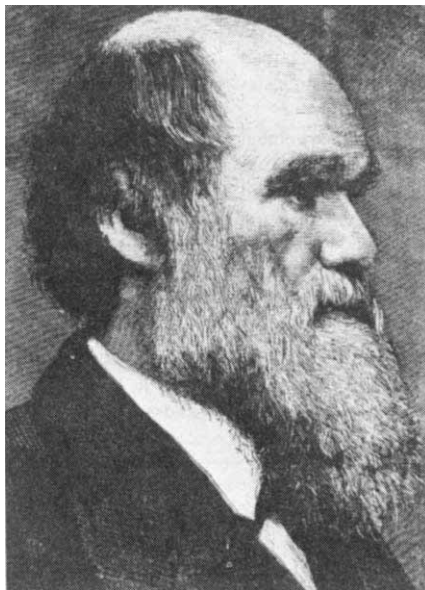
LUDWIG BOLTZMANN once wrote that the nineteenth century would be remembered as the century of Darwin. One hundred years after Darwin's death this judgement still seems perceptive. No other writer had such a profound effect on the way we see ourselves, and no other brought about so great an extension in the range of subjects which we regard as explicable by scientific theory. Here, however, I shall confine myself to his contribution to evolutionary biology, and shall forget that he was the founder of ecology and ethology, and made significant contributions to geology and psychology. It was, after all, his formulation of the theory of evolution by natural selection that was decisive.

In recent years there have been claims — in the daily press, on television, and by retired cosmologists — that Darwin may have got it wrong. Some excuse can be found in the fact that Darwin has indeed been criticized by scientists working in a variety of fields — for example palaeontology, taxonomy and embryology. At least one group of scientists has claimed that a new evolutionary paradigm is on the way. However, to see Darwinism as being under serious threat would, I think, be a false perception. The error arises because Darwin's theory is so central to modern biology that any new idea may first be seen (as Mendelian genetics was seen) as being in conflict with Darwinism.

Let me first give a brief history of evolutionary ideas since Darwin. In the *Origin of Species*, Darwin aimed to establish two things. First, he argued that evolution had in fact happened (that is, that all existing organisms are descended from one or a few simple ancestral forms), and, second, that the main cause of evolutionary change was the natural selection of variations that were in their origin non-adaptive. The main weaknesses of his position were that he had no adequate theory of genetics, and that he could give no satisfactory account of the origin of the variations on which selection would later act. In genetics, Darwin was a Lamarckist. That is, he thought that if organisms acquired characteristics by use and disuse during their lifetimes, this would influence the nature of their offspring. In thinking this, he was sharing an opinion held by almost all his contemporaries.

The first major advance after Darwin was made by August Weismann, who

argued for the independence of the 'germ line' (the cell lineage leading from the fertilized egg to the germ cells, egg and sperm, which form the starting point of the next generation) from the 'soma' (the cell lineage from fertilized egg to adult body). As a boy, I acquired, from reading the preface to Shaw's *Back to Methuselah*, a picture of Weismann as a cruel and ignorant German pedant who cut the tails off mice to see if their offspring had tails. What a ridiculous experiment! Since the



mice did not actively suppress their tails as an adaptation to their environment, no Lamarckist would expect the loss to be inherited. Much later, I discovered that Weismann was not as I had imagined him. His experiment on mice was performed only because, when he first put forward his theory, he was met with the objection that, (as was, it was claimed, well known) if a dog's tail is docked, its children are often tailless — an early use of what J.B.S. Haldane once called Aunt Jobisca's theorem, 'It's a fact the whole world knows'.

Much more interesting are Weismann's reasons for proposing his theory, and its implications for Darwinism. At first sight, his reasons were poor. It is often not the case (for example, in higher plants) that the germ line is a lineage distinct from the soma. Even when it is distinct, the material

and energy it needs are supplied by the soma. In Weismann's day, the experimental evidence for the 'non-inheritance of acquired characters' was weak. Why, then, did he believe it? I think that the clue lies in his remark that, if one were to come across a case of the inheritance of an acquired character, it would be as if one were to send a telegram to China and it arrived translated into Chinese. This is the first use known to me of the information analogy in heredity. Weismann did not accept the inheritance of acquired characters because he could not conceive of a mechanism of 'reverse translation', whereby the hypertrophied muscles of the blacksmith could be translated into genes (he called them 'ids') which could, in the next generation, cause the growth of large muscles.

If Weismann was right, this greatly strengthened Darwin's theory. Natural selection, instead of being just one of the possible processes leading to evolutionary adaptation, becomes the only process (at least, until the evolution of organisms sufficiently intelligent to learn from their parents).

The next important step was the rediscovery of Mendel's laws at the start of this century, and the formulation of the chromosome theory of heredity. The first impact of Mendelism on evolutionary biology was distinctly odd. The early Mendelians saw themselves as anti-Darwinians; Darwin's banner was held aloft by the biometric school, who concentrated on measuring the correlations between relatives, and who regarded genes as metaphysical entities. The Mendelians saw the 'mutations' they studied as each being the potential starting point of new species, and the continuous variation studied by the biometricians as evolutionarily irrelevant; the biometricians saw mutations as pathological deviants doomed to early elimination by selection, and continuous variation as the stuff of evolution. The argument, led by Bateson on the one side, and Pearson on the other, foreshadowed the current debate between punctuationalists and gradualists. It is part of the larger debate between those who see the world as continuous and those who think it proceeds in jerks.

The debate, at least in the form in which it then presented itself, was largely settled by the work of the population geneticists,

NATURE

THURSDAY, APRIL 27, 1882

CHARLES DARWIN

VERY few, even among those who have taken the keenest interest in the progress of the revolution in natural knowledge set afoot by the publication of the "Origin of Species"; and who have watched, not without astonishment, the rapid and complete change which has been effected both inside and outside the boundaries of the scientific world in the attitude of men's minds towards the doctrines which are expounded in that great work, can have been prepared for the extraordinary manifestation of affectionate regard for the man, and of profound reverence for the philosopher, which followed the announcement, on Thursday last, of the death of Mr Darwin.

Not only in these islands, where so many have felt the fascination of personal contact with an intellect which had no superior, and with a character which was even nobler than the intellect; but, in all parts of the civilised world, it would seem that those whose business it is to feel the pulse of nations and to know what interests the masses of mankind, were well aware that thousands of their readers would think the world the poorer for Darwin's death, and would dwell with eager interest upon every incident of his history. In France, in Germany, in Austro-Hungary, in Italy, in the United States, writers of all shades of opinion, for once unanimous, have paid a willing tribute to the worth of our great countryman, ignored in life by the official representatives of the kingdom, but laid in death among his peers in Westminster Abbey by the will of the intelligence of the nation.

It is not for us to allude to the sacred sorrows of the bereaved home at Down; but it is no secret that, outside that domestic group, there are many to whom Mr Darwin's death is a wholly irreparable loss. And this not merely because of his wonderfully genial, simple, and generous nature; his cheerful and animated conversation, and the infinite variety and accuracy of his information; but because the more one knew of him, the more he seemed the incorporated ideal of a man of science. Acute as were his reasoning powers, vast as was his knowledge, marvellous as was his tenacious industry, under physical difficulties which would have converted nine men out of ten into aimless invalids; it was not these qualities, great as they were, which

impressed those who were admitted to his intimacy with involuntary veneration, but a certain intense and almost passionate honesty by which all his thoughts and actions were irradiated, as by a central fire.

It was this rarest and greatest of endowments which kept his vivid imagination and great speculative powers within due bounds; which compelled him to undertake the prodigious labours of original investigation and of reading, upon which his published works are based; which made him accept criticisms and suggestions from any body and every body, not only without impatience, but with expressions of gratitude sometimes almost comically in excess of their value; which led him to allow neither himself nor others to be deceived by phrases, and to spare neither time nor pains in order to obtain clear and distinct ideas upon every topic with which he occupied himself.

One could not converse with Darwin without being reminded of Socrates. There was the same desire to find some one wiser than himself; the same belief in the sovereignty of reason; the same ready humour; the same sympathetic interest in all the ways and works of men. But instead of turning away from the problems of nature as hopelessly insoluble, our modern philosopher devoted his whole life to attacking them in the spirit of Heraclitus and of Democritus, with results which are as the substance of which their speculations were anticipatory shadows.

The due appreciation or even enumeration of these results is neither practicable nor desirable at this moment. There is a time for all things — a time for glorying in our ever-extending conquests over the realm of nature, and a time for mourning over the heroes who have led us to victory.

None have fought better, and none have been more fortunate than Charles Darwin. He found a great truth, trodden under foot, reviled by bigots, and ridiculed by all the world; he lived long enough to see it, chiefly by his own efforts, irrefragably established in science, inseparably incorporated with the common thoughts of men, and only hated and feared by those who would revile, but dare not. What shall a man desire more than this? Once more the image of Socrates rises unbidden, and the noble peroration of the "Apology" rings in our ears as if it were Charles Darwin's farewell:—

"The hour of departure has arrived, and we go our ways — I to die and you to live. Which is the better, God only knows." T.H. HUXLEY

Fisher, Haldane and Wright. Two points were made clear. First, the continuous variation studied by the biometricians could be explained by alternative alleles at many loci, each by itself having a small effect on the phenotype. Second, even rather small differences in fitness between genotypes are sufficient to determine the direction of evolutionary change, despite mutation being mainly in an opposition direction.

The work of the population geneticists prepared the way for the 'modern synthesis' of evolutionary biology, developed in the period 1930–1950 by a group including Dobzhansky, Ford, Julian Huxley, Mayr, Muller, Rensch, Simpson and Stebbins. It is hard in a few sentences to describe what these men did. In effect, they showed that the 'neo-Darwinian' mechanism — natural selection in Mendelian populations — was sufficient to explain the evolutionary process as it could be observed in nature. Dobzhansky, Ford and others measured genetic variability and natural selection in wild populations.

Mayr and Rensch (for animals) and Stebbins (for plants) studied geographical variation within and between species, and discussed how new species might arise. Simpson argued that the fossil record could best be understood in Darwinian terms. Most research in evolutionary biology since that time has been carried out in the framework of the modern synthesis. Particular efforts have been made in areas which, at least at first sight, seem to be difficult to explain in terms of natural selection, for example, the evolution of social behaviour and of sex and breeding systems.

Since 1950, developments in molecular biology have had a growing influence on the theory of evolution. The 'central dogma' of molecular biology, according to which information can pass from nucleic acid to protein, but not from protein to nucleic acid, provides a molecular explanation for Weismann's principle, thus leaving natural selection as the only agent of adaptation. Important as this is, however, two additional points should be

made. First, even if 'reverse translation' of amino acid sequences into base sequences were possible, this would not provide a general mechanism for Lamarckian inheritance, because most developmental adaptations do not involve the production of new protein sequences. Second, there are good reasons why, even if living organisms have arisen independently many times in the universe, Lamarckian processes should play a minor role in their evolution. Most 'acquired characters' are non-adaptive — they are the results of age, injury and disease. Therefore, a hereditary mechanism which transmitted such characters to offspring would work against the evolution of adaptation. Hence the one-way flow of information from nucleic acid to protein may have been a necessary feature of an hereditary mechanism able to support evolution. In physics, the second law of thermo-dynamics asserts that entropy will increase in a closed physical system. In biology, Weismann's principle, together with the principle of natural selection, makes possible the maintenance,

and even the increase, of information in open biological systems.

Molecular biology has had an impact on evolutionary theory in other ways. Protein electrophoresis has provided a way of measuring the genetic variability of populations; its main value may be in enabling us to discover more about the breeding structure of populations. Sequence data on proteins and nucleic acids can be used to work out the phylogenetic relationships of existing organisms. In this context, sequences have the advantage over morphological data in that they provide a means of estimating the number of genetic changes separating two forms. The information we are acquiring about how DNA is arranged in chromosomes may at last give us some insight into the evolutionary significance of chromosome structure. Two questions in particular are being asked. First, does the large-scale arrangement of genes on chromosomes have any significance for development, or is it merely a way of ensuring accurate gene segregation during cell division? Second, does all the DNA in the genome perform some useful function in the survival or reproduction of the organism, or is some part of it 'selfish' or 'parasitic'? The second question raises a set of problems which are logically similar to those which have been debated for some time by students of the evolution of social behaviour, that is, questions about the levels at which selection acts and the differences between what Dawkins has called 'replicators' and 'vehicles'.

There is, however, one area of molecular biology which seems to me to lag behind the rest. This is the study of the evolution of prokaryotes (organisms such as bacteria lacking a proper cell nucleus). The modern synthesis of the 1940s was concerned with eukaryotes (organisms with a cell nucleus, usually sexual and diploid). Its essential achievement was to bring together two previously separate disciplines — the chromosome theory of heredity and the study of natural populations. The same synthesis is now required for the prokaryotes. There is an abundant knowledge of their genetics, but as yet no adequate synthesis of that knowledge with a study of the natural history of bacteria. For example, we have little idea of the significance of conjugation for bacterial populations; it is as if we had no idea of the significance of sexual reproduction for populations of birds or insects. Population thinking has been well developed for fully half a century, but has yet to be adopted by microbiology. □

This essay is an abbreviated version of the introduction to *Evolution Now*, a collection of recent papers on controversial issues in evolution with commentaries by John Maynard-Smith. *Evolution Now* is being published by Macmillan Press and, in the USA, by W.H. Freeman. It will be available to *Nature* subscribers as advertised in next week's issue.

The Times,
3 May 1982

THE LATE MR. DARWIN.

At the scientific meeting of the Zoological Society, yesterday evening, before the commencement of the proceedings, the president, Professor Flower said,—"The minutes read recall the fact that at our last meeting we were honoured by a communication from Dr. Darwin, probably his last contribution to that science to which he devoted his life-long labours. No one who heard that paper, showing as it did no sign of faltering from that eager interest which he had always manifested in a subject which he had made peculiarly his own, expected that not 24 hours would elapse before those labours would be brought to a close. During the fortnight that has passed the whole world has been moved at the loss it has sustained, and his work and his character have, more than any other theme, filled the minds of thinking people of all countries, classes, creeds, and occupations. We who humbly follow him in cultivating the science he adorned must feel elevated at the sight of the full recognition accorded to his work. The general acceptance of Darwin as one who has exercised a powerful influence upon the whole realm of human thought, the cordial reception of his remains in our magnificent Abbey, among the illustrious men of whom our country is proud, are triumphs in the history of zoology, for it was mainly zoological observation which led to those philosophical speculations which have made his name famous. The nation's grief at his loss has already found eloquent and feeling expression in many quarters; the resources of our language seem to have been exhausted in bearing testimony to his worth. No words that I could find would add anything to what has been so well said by others; and surely here, if in any place in the world, among those who are always occupied with subjects the pursuit of which has been so profoundly modified by his writings, and among many who loved him as a personal friend, nothing is needed but to mention his name to call forth the strongest feelings of admiration for his work and reverence for character. If it is not given to any of us to emulate him in brilliancy of scientific induction, or to light upon discoveries that will change the current of human ideas, we can at least endeavour to follow the example he has set us of patient perseverance in observation, scrupulous accuracy of statement, deference for the opinions and feelings of others, candour towards opponents, and of that invariable modesty and gentleness of demeanour which shed such a charm around his public as well as his private life.

LATEST INTELLIGENCE

(FROM OUR CORRESPONDENTS.)

(BY TELEGRAPH.)
FRANCE.

PARIS, MONDAY, APRIL 24.

Mr. Darwin has to be added to the long list of eminent men, not excluding Catholic prelates, whom the *Univers* has denounced or decried. Its wrath has been excited by the praises of the *Radical Justice*. The notices in the French Press, by the way, have mostly been very meagre. It thus winds up two columns of sarcasm:—

"When hypotheses tend to nothing less than the destruction of faith, the shutting out of God from the heart of man, and the diffusion of the filthy leprosy of Materialism, the *savant* who invents and propagates them is either a criminal or a fool. *Viola ce que nous avons à dire du Darwin des singes.*"

The Times, 25 April

TO THE EDITOR OF THE TIMES

Sir,—May I beg a corner for my feeble testimony to the marvellous persevering endurance in the cause of science of that great naturalist, my old and lost friend, Mr. Charles Darwin, whose remains are so very justly to be honoured with a resting-place in Westminster Abbey?

Perhaps no can better testify to his early and most trying labours than myself. We worked together for several years at the same table in the poop cabin of the *Beagle* during her celebrated voyage, he with his microscope and myself at the charts. It was often a very lively end of the little craft, and distressingly so to my old friend, who suffered greatly from sea-sickness. After, perhaps, an hour's work he would say to me, "Old fellow, I must take the horizontal for it," that being the best relief position from ship motion; a stretch out on one side of the table for some time would enable him to resume his labours for awhile, when he had again to lie down.

It was distressing to witness this early sacrifice of Mr. Darwin's health, who ever afterwards seriously felt the ill-effects of the *Beagle's* voyage.

I am, Sir, yours truly,

J. LORT STOKES, Admiral.

Scotchtwell, Pembrokeshire, April 25.

The Times, 27 April

CHARLES ROBERT DARWIN

Exactly a year to a day has separated the deaths of two of the most powerful men of this century, some have said of any century; and those who care for the task will find some very curious analogies between the progress and the ultimate results of the work of the two men, totally different as were the spheres in which they exercised their remarkable powers. On April 19, 1881, all the civilized world held its breath at the news of the death of Lord Beaconsfield; not less must be the effect upon the most civilized part of the civilized world when the announcement of the death of Charles Darwin flashes over the face of that earth whose secrets he has done more than any other to reveal. All who knew anything of Mr. Darwin know that, massive as he seemed, it was only by the greatest care and the simplest habits that he was able to maintain a moderate amount of health and strength. Mr. Darwin had been suffering for some time past from weakness of the heart, but had continued to do a slight amount of experimental work up to the last. He was taken ill on the night of Tuesday last, when he had an attack of pain in the chest with faintness and nausea. The latter lasted with more or less intermission during Wednesday and culminated in his death, which took place at about 4 o'clock on Wednesday afternoon. He remained fully conscious to within a quarter of an hour of his death. His wife and several of his children were present at the closing scene. During his illness he had been attended by Dr. Norman Moore, Dr. Andrew Clarke, Dr. Moxon, and Dr. Alfrey, of St. Mary Cray.

Fifteen volumes lie before us and nearly as many memoirs large and small, the product of 45 years' work — a product which, in quantity, would do credit to the most robust constitution. But when we consider Mr. Darwin's always feeble health and his deliberately slow method of work, never hasting but rarely resting, the result seems marvellous. But wonderful as this is under the circumstances, it is not by mere quantity that Mr. Darwin's work will be judged: the quantity is of chief importance in respect of the multifarious channels through which his influence has spread.

The Times, 21 April

NEWS AND VIEWS

Methylation and gene control

from Gary Felsenfeld and James McGhee

ARE changes in DNA methylation patterns a cause or an effect of eukaryotic gene expression? There are already many examples of an inverse correlation between the level of methylation in the neighbourhood of a gene and the transcriptional activity of that gene. It is more difficult to prove that the extent of methylation is in itself sufficient to determine gene activity.

Several recent studies tackle this problem by making use of the inability of bacterial restriction endonucleases to cleave DNA restriction sites that have been methylated. In eukaryotes, the site of methylation is almost invariably the 5' position of cytosine and, in animals, the cytosine residue is usually contained in the sequence CpG. The restriction endonucleases *HpaII* and *MspI*, which can cut the sequence CCGG, have therefore proved useful tools in these studies¹. Methylation renders the sequence resistant to the *HpaII* endonuclease, but not to *MspI*, and with the use of gel electrophoresis and Southern blotting, the pattern of methylation at those CpG sites which are embedded in a CCGG sequence can be determined in some detail.

It has been shown with these (and other) restriction enzymes that certain CpG sites in the vicinity of a wide variety of genes are undermethylated in a tissue in which the gene is expressed compared with a tissue in which the gene is inactive (reviewed in ref. 2). Unfortunately, only a fraction of methylation sites are also restriction sites and can be detected in this way. The pattern of variation in methylation is complex, and usually does not involve complete demethylation of extensive regions of the DNA, although the pattern tends to be stably inherited within a given cell line. This is presumably because the enzyme bringing about methylation acts much more efficiently on hemimethylated sites than on unmethylated ones, and therefore methylates the daughter strand of a newly replicated duplex wherever the parent strand is methylated.

To show that DNA methylation directly affects gene expression requires an experimental method of altering methylation levels. Recently the *HpaII* methylase has been used to introduce methyl groups into defined DNA sequences. In early studies^{3,4}, the herpes thymidine kinase (*tk*) gene was methylated *in vitro* and used to transform mouse *Ltk*-

cells. Methylation decreased the transformation efficiency, and transformants selected for *tk* expression carried *tk* sequences that were partially demethylated relative to the input DNA. Though these results are compatible with an inhibitory effect of methylation on *tk* expression, their interpretation is complicated by the possible effects of methylation on the transformation process itself. Such effects cannot be measured because apparently only cells carrying at least partially demethylated *tk* sequences survive.

A new experiment by Stein *et al.*⁵ solves the problem by using separate genes for selection and for methylation. Mouse *Ltk-aprt* cells were transformed with herpes *tk* DNA, and in addition co-transformed with hamster *aprt* (adenine phosphoribosyl transferase) DNA. The *aprt* gene was introduced either in the unmethylated form or after methylation *in vitro* by *HpaII* methylase. Clones selected for *tk*⁺ activity were then assayed for expression of the co-transformed but unselected *aprt* gene. (It has been shown earlier⁶ that methylation patterns of transforming sequences not subject to selection pressure are stable over many generations.) Clones transformed with unmethylated *aprt* sequences were found to express the *aprt* gene. Clones transformed with methylated *aprt* neither transcribed nor expressed the gene, even though the methylated sequences could be detected within the transformed cells by Southern blotting, and appeared to be incorporated into the genome with an efficiency similar to that for unmethylated *aprt* sequences. When the clones carrying methylated *aprt* were subjected to conditions selecting for the *aprt*⁺ phenotype, reversion was found to be associated with undermethylation of the *aprt* sequences. The authors conclude that the observed effect of methylation on *aprt* activity arises from a direct influence of methylation on gene expression.

The connection between DNA methylation and gene expression is supported by studies of mouse retroviruses. A number of workers had previously shown that the inactive, gene-

tically acquired proviral sequences are heavily methylated whereas the active, somatically acquired copies are undermethylated. In a recent study, Stuhlmann *et al.*⁷ used transfection of cell cultures by purified mouse DNA to show that the nonmethylated proviral sequences were infectious whereas the methylated sequences were not. Furthermore, a copy of the endogenous inactive provirus was cloned in bacteria and the cloned DNA (now unmethylated at CpG sites) was found to be highly infectious⁸.

A possible objection to DNA transfection and transformation studies is that cytosine methylation could direct the site of DNA integration. For example, methylated DNA could integrate into methylated regions of the chromosome, which are inactive for other reasons. Perhaps this objection could be overcome by more detailed studies of flanking regions or by transformation with methylated and unmethylated sequences ligated together in the same molecule. In any case, a methyl-directed integration model cannot explain the results of Vardimon *et al.*⁹. A region of adenovirus coding for the *Ad2*-specific DNA binding protein was cloned and the DNA, either left unmethylated or methylated *in vitro* with the *HpaII* methylase, was injected into *Xenopus* oocytes. The results were striking: unmethylated DNA produced adenovirus-specific RNA, whereas the methylated DNA did not.

Levels of methylation can also be deliberately altered by the use of the nucleoside analogue 5-azacytidine (5-azaC), which inhibits DNA methylation *in vivo*. Jones and Taylor¹⁰ have shown that 5-azaC can induce differentiation in cultured mouse embryo cells, and that at the same time and dosage the cellular DNA becomes demethylated. Three other cytidine analogues behave similarly, whereas two more that do not induce differentiation do not affect methylation. Two recent studies have extended this approach to an analysis of the state of individual genes after cell growth in 5-azaC. Groudine *et al.*¹¹ report that 5-azaC induces expression of an endogenous avian retrovirus, and that this expression is associated with both DNA demethylation and increased nuclease sensitivity of the viral sequences in chromatin. Compere and Palmier¹² have studied the effect of 5-azaC on the

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expression of the metallothionein gene in a mouse thymoma cell line in which the gene is normally uninducible. After exposure to 5-azaC, sites on the gene are unmethylated and the gene becomes inducible. Finally, 5-azaC has been used to enhance the reactivation of the inactivated human X chromosome in mouse-human cell hybrids¹³, an effect consistent with the proposal that DNA methylation is the basis of X chromosome inactivation in female mammals^{13,14}.

The mechanism by which 5-azaC causes demethylation is unclear. The analogue apparently must be incorporated into DNA to inhibit methylation^{10,12}, but it cannot function merely by virtue of a lack of a methylatable atom in the 5 position, since the extent of demethylation far exceeds the amount of 5-azaC incorporation. Furthermore, not all genes can be activated by non-lethal concentrations of 5-azaC^{5,15}, perhaps because activation of some genes requires more extensive demethylation than can be achieved with non-toxic levels of 5-azaC. There is evidence that methylase can act as a processive enzyme, so that a single 5-azaC in a daughter DNA strand could affect the methylation of cytosines downstream on that strand. If binding of methylase *in vivo* were coupled to replication, the effect of 5-azaC on gene expression could be a complicated function of the distance between the methylatable control region of the gene and the nearest replication origin.

Although methylation levels are clearly correlated with expression in the cases discussed above, there are some genes, even in organisms with a high general level of DNA methylation, that do not display such obvious correlations. For example, the $\alpha(2)$ I collagen gene of chicken has a pattern of *HpaII* site methylation that is invariant (with some sites completely unmethylated) in five cell types that have been studied, whether or not the cell type synthesizes detectable amounts of collagen¹⁶. Another anomaly has been reported in the case of X chromosomes from normal human cells, which in contrast to X chromosomes from mouse-human cell hybrids reveal no simple correlation between chromosome inactivation and the pattern of methylation at *HpaII* sites. Although these may be examples of situations in which control of expression is not coupled to altered levels of methylation, it is also possible that the crucial methylation sites are not *HpaII* sites.

In any case, these results are not inconsistent with models in which absence of methylation at specific residues is necessary for gene expression. Analysis of existing data suggests that the methylation sites critical to a given gene's control processes may be a small fraction of those sites present in that DNA region. The effect of methylating these critical sites presumably is to alter the way in which proteins interact with the region, either by

modifying local interactions with the active sites of sequence-specific proteins, or by longer-range effects on DNA structure. In the synthetic polynucleotide poly(dC-dG). poly(dC-dG), which consists entirely of CpG repeats, methylation of the cytosine residues causes the polymer to adopt the left-handed Z DNA conformation under physiological salt conditions¹⁷. Furthermore, the Z form has so far proven incapable of forming nucleosomes¹⁸. Such results lead quite naturally to the idea that methylation *in vivo* may have a major effect on DNA and chromatin structure, but one should not try to be too specific, at this stage, concerning the nature of such effects. There is, for example, at the 5' end of the chicken adult β globin gene a region 200 base pairs long, which contains about six times the genomic frequency of CpG sites, and which is exposed, apparently nucleosome free and reduced in methylation when the globin gene is active¹⁹. Examination of the nucleotide sequence does not, however, reveal any extended sequence of alternating purines and pyrimidines of the kind required for Z DNA formation, so that the effects (if any) of methylation on the structure of this region are likely to be more subtle.

Although it now appears that DNA methylation plays an important role in gene expression during development, it should

be recalled that some organisms (notably insects) manage quite well without any extensive methylation. The mechanisms for marking expressed genes in such organisms, like the mechanism that marks genes for demethylation in the organisms discussed above, remains unknown. □

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Vitamin A, limb patterns and the search for the positional code

from Jonathan Cooke

By around 1940 such figures as Boveri, Harrison, Child and Spemann had propounded, in essence, all the rival scientific theories that we have subsequently lived with concerning animal morphogenesis, with its reliable and typical spatial patterns of cellular differentiation. There has followed a wealth of phenomenological work fleshing out one or another of these theories, and resulting in refinement or renaming of ideas according to the allegiances of the workers concerned.

But this has been accompanied by so little progress towards real knowledge of the biological machinery of development that some scientists see the efforts of the last forty years as taking place in a world like that of Herman Hesse's allegorical novel 'The Glass Bead Game', where compulsive scholars are forever playing a purely internal and, by pre-arrangement, interminable game with the pieces of theories. A sour-minded view maybe, but the sad fact is that the various conjectures about morphogenetic mechanisms have swung in and out of fashion over the years in a way that would be more appropriately explained by micro-

sociologists or social historians of science than by the scientists themselves.

As an addicted insider I report the embryological glass bead game to be still alive and compulsively enjoyable, but can nevertheless see the present force of argument for a swing towards a more reductionist, biochemical approach to promote real progress. An opportunity for just this may be presented by two exciting new reports that compounds of the vitamin A family exert systematic effects upon the anatomical patterns of developing and regenerating limbs. The effects are separable from uninformative toxicity, and involve formation of elements of the familiar patterns that are in themselves histologically normal, but in quite abnormal spatial configurations.

In one case Maden¹ shows that regeneration from the amputated limb stump of an amphibian is disturbed when its early phases are delayed by the presence of retinoic acid or its derivatives in the bathing medium. The terminal population of locally de-differentiated cells or

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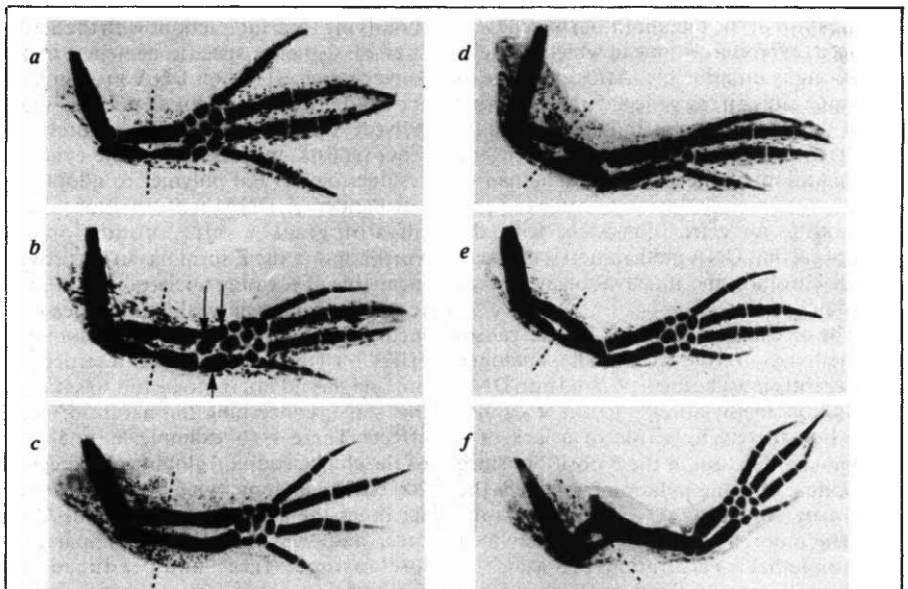
blastema, from which the regenerate develops, normally differentiates to replace just that part of the base-to-tip sequence that was removed by amputation. In Maden's experimental cases, it reiterates an inappropriately large part of this base-to-tip sequence, so that the limb finally regenerated has internal pattern repetition in the long axis. The precise level in the pattern from which the blastema cells now re-commence differentiation, and thus the extent of the final repetition, depends largely on the level of amputation in the original limb and the length of exposure to the vitamin over several days, rather than being a simple expression of the chemical concentration of the agent. Neither delay in initiation of regeneration due to other agencies nor, indeed, any other known manipulation can effect such 'proximalisation' of the positional values recorded in these blastema cells.

In Maden's words, these results "all contravene the rule of distal transformation", a rule that was coined to express the phenomenon that biological patterns seem characterized by an 'uphill-to-downhill' polarity, such that regeneration by cells at a cut edge will normally proceed only downhill. The fact that a novel experimental circumstance, and especially a biochemical insult, can by-pass such 'rules' should not, however, surprise us. Organisms consist of evolved arrangements whereby normal processes and results have been assured given normal surrounding circumstances, whereas the pre-biotic world, so far as we know, does not. Thus while Newton might well stir momentarily should two asteroids be observed to jink away from each other as they passed in their orbits, no one coining a 'rule' or 'law' as part of a theory for biological process expects it to be proof against all future insults.

In the other case, Tickle *et al.* (*Nature*, in the press) show that early development of the bird limb bud is disturbed by implantation of a local reservoir of retinoic acid in an *anterior position* within it. But here, the systematic mirror duplications in the antero-posterior axis of pattern, that is, the sequence of digits that are produced, are similar to the result of implanting a particular piece of indigenous tissue — the normally posteriorly situated zone of polarizing activity — to a similar anterior position.

Even a speculative comparison of the vitamin A concentrations experienced by the reacting cells in the two systems is difficult, but in the bird experiments², the local concentration near the source, and perhaps in the entire limb, may be an order of magnitude higher than the upper end of the ambient concentration range in the regeneration study¹. The length of time for which it is present in the limb buds, in relation to the days-long exposure of the blastemas, is unknown (or at least not stated).

It is, at first, astounding that separate



The limbs shown above regenerated after amputation through the mid-radius and ulna (broken line). The distal radius, and ulna, carpals and digits have all been replaced. (a) A control limb which regenerated in pure tap water (b-e) Limbs treated with 15 IU ml⁻¹ retinol palmitate for 12 days. In each case pattern elements were added; (b) contains extra carpal-like cartilage; (c) an abnormal length of radius and ulna; (d) a complete extra radius and ulna and (e) duplication as far back as the distal end of the humerus and including a perfect elbow joint. In (f) a complete limb has been produced, distal to the amputation plane, by exposure to a higher concentration of retinol palmitate (15 days in 75 IU ml⁻¹).

Increasing the concentration or time of exposure to retinol palmitate causes the level from which the limb was repeated to become more and more proximal until finally a complete limb is regenerated from the amputation plane. When the position of the amputation plane is varied it is found that higher levels of retinol palmitate are needed to regenerate complete limbs from more distal positions; only the highest concentration at the longest time produced regeneration of a complete limb from the carpal level.

dimensions of the anatomical pattern are exclusively affected in each system, when they are, after all, homologous episodes of pattern formation in related organisms. The entire enterprises of comparative anatomy and causal embryology can, however, be rescued if one chooses to deduce, from other data available, that pattern axes in the present experiments are affected according to whether or not, in each system, certain spatial interactions are possible along them (that is, whether they are in what embryologists refer to as the labile or the mosaic state) at the time of exposure.

Unpublished work at the National Institute of Medical Research suggests that ambient exposure, rather than localised sources, in the chick limb, affects the antero-posterior pattern axis, which is the labile one at the time, but does not cause duplications. So one very abstract scheme would be that proximal and posterior boundaries of the pattern represent 'source' or 'origin' states, and that excess vitamin drives cells progressively and artificially towards these regardless of the state previously dictated by their position. During limb outgrowth, the proximo-distal sequence of pattern contributions evolves locally in the cell population of the tip by means of time itself, time which can be 'set back' by means of simple exposure to the compound. In anterior-posterior pattern formation in the chick limb, by contrast,

polarity has normally to be maintained across a zone of tissue one millimetre wide where the cells are still labile. This is accomplished by prolonged signalling, from a localized source, the zone of polarizing activity. Hence the requirement for a localized reservoir of the experimental agent to provoke a new boundary elsewhere and thus duplication.

But almost nothing can be deduced from the present observations simply by plugging them into current detailed but abstract models, involving such entities as separable cell positional values and 'interpretation' processes, pre-patterns, progress zones, serial inductions and so on (see for example, refs 3-5). All such schemes are, at best, simply consistent with, rather than positively dictated, by the phenomena they have sought to explain, and with the hindsight of final understanding they might even come to be seen as having impeded, rather than helped, attempts to understand effects such as that of retinoic acid.

Anyone who would make sense of the new phenomena had better be prepared to be involved with protein gels, fractionation columns and membrane preparations, for surely the genuine excitement is in the promise they hold out for biochemical investigation of pattern control in appropriate experimental material, via our imperfect knowledge of the loci at which vitamin A inserts itself into the metabolic

functions of the cell. One can only hope for sustained effort rewarded by good luck in any such assisted guessing games, for it would be sad if the present results fared no better than the 'lithium phenomenon'.

Lithium is the best known of a small, chemically diverse group of simple substances, long ago discovered to have systematic distorting effects, this time on primary proportioning of the whole body pattern at its first determination, in ways which are 'homologous' in a wide variety of embryo types. The effects are of the same order of specificity as the present ones. They must be telling us something about the biochemistry or cell biology of spatial organization^{6,7}, but no-one has ever discovered what. Lithium ions, substituting for, and thus distorting, the role of sodium as they presumably do, may affect so many membrane and other

functions that the domain to be searched for the relevant effect is very great. We might hope for more success with retinoic acid with its more restricted list of functions. Mediation in the glycosylation of proteins, effects upon gap junction formation and upon the balance of secreted matrix proteins have been mentioned in the publications. These molecular assemblies have each been previously suggested as candidates for involvement in the setting up or maintenance of tissue positional codes. □

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T cells immortalized?

from Nigel Williams

SUCCESSFULLY fuse an antibody-producing B cell with a myeloma cell and there is a good chance that the resulting hybridoma will provide a continuous supply of monoclonal antibodies. Can similar techniques be applied to T cells, the other major population of lymphocytes within the immune system, whose function is much less well understood? The answer that emerged from a recent meeting in Basel* is that in many cases it can. T cells are a functionally heterogeneous population of cells whose antigen-specific surface or soluble receptor molecules remain a puzzle, so the production of functional T-cell hybridomas to provide a monoclonal source is clearly of great interest. How these molecules relate to antibodies and how many different molecules are produced are two major questions to which the production of hybridomas has been addressed.

The function of one major population of T cells is to help B cells produce antibodies. On stimulation by antigen presented by macrophages bearing self molecules by the major histocompatibility complex (MHC), T cells can release molecules which help B cells produce antibodies against the same antigen. However, from cloning such T cells it is clear that on stimulation they can also release other factors. Interleukin-2, also known as T-cell growth factor (TCGF), is the best characterized and can stimulate the growth of resting T cells. Stimulated helper T cells can also provide bystander help, that is, they can stimulate antibody production by B cells regardless of their specificity. How this

function is achieved is not understood.

M. Schreier (Basel Institute for Immunology) has produced clones of mouse helper T cells generated against egg albumin (EA). While all the clones could provide all these functions, the amount of help for antibody responses against EA and TCGF production varied greatly between different clones and neither of these functions correlated with the amount of bystander help. He has produced hybridomas from these cloned T cells which also preferentially expressed different functions. Although it is not yet clear how far these differences in helper function result from quantitative differences in the production of similar molecules, and how far from differences in the molecules produced, stable factor-producing hybridoma clones should eventually help resolve these questions.

Many of the characteristics of mouse helper T cells have been confirmed in human ones. E. de Freitas (Wistar Institute, Philadelphia) has produced hybridomas between human helper T cells generated against tetanus toxoid and a human T-lymphoma cell line which produce TCGF and also help B cells produce antibodies against tetanus toxoid. As in mice, the expression of these two functions varied in different hybridoma clones. It is not known whether these cells can also produce bystander help but if they do, then the exciting possibility is raised that human antibodies could readily be produced *in vitro*.

A second population of T cells functions

to suppress antibody production. Hybridomas have provided a monoclonal source of the molecules involved. Although, like antibodies, these molecules carry an antigen-binding component, some also appear to carry determinants encoded by the I region of the MHC. However it is not clear whether antigen binding and I-region determinants are found on a single polypeptide chain.

M. Taniguchi (Chiba University) described recent work on hybridoma clones producing a suppressor factor specific for the antigen keyhole limpet haemocyanin (KLH), expressing I-J-region determinants. This could be extracted from the hybridoma cells or obtained in the ascites from hybridoma-bearing mice as a secreted factor. By use of immunoabsorbent columns he showed that the activity of the extracted factor could be reconstituted by mixing equal proportions of eluates from an anti-I-J column and a KLH column. If the secreted factor was first subjected to reducing conditions, similar reconstitution of activity could be obtained by mixing eluates from these two columns. This evidence for a two-chain factor is supported by experiments in which mRNA extracted from the hybridomas was injected into *Xenopus* oocytes where it was translated. The mixture of equal quantities of two translocation products showed strong suppressor activity with KLH specificity, while either product alone was without effect.

D. Webb (Roche Institute of Molecular Biology, Nutley) has also produced T-suppressor cell hybridomas, but specific for the synthetic polypeptide GAT, in two different strains of mice. One of these strains can respond and produce antibody following challenge with the antigen, whereas the other (non-responder) strain is unable to produce anti-GAT antibody. However specific suppressor T-cell releasing factors can be found in both mice. These have been highly purified from the hybridomas and one has recently been reported (*Proc. natn. Acad. Sci. U.S.A.* **97**, 1254; 1982). (Such a feat demonstrates the importance of a monoclonal source — to obtain 2 mg of factor, 10 litres of supernatant were required. Slightly better yields were obtained from the extracted factor.)

It has been shown that these molecules have an antigen-binding region and carry I-J-region-encoded determinants. However it seems that the factor from the non-responder mice is a single polypeptide while in the responder strain there are two different factors: one a single-chain and the other a two-chain polypeptide. Cloned cDNA encoding these molecules has now been produced and should provide further illumination.

In another system where suppressor T cells control the contact sensitivity response to the hapten nitrophenol, M. Dorf (Harvard University) described a series of three interacting suppressor cell

*A meeting on T-cell hybridomas was organized by and held at the Basel Institute for Immunology, 27–29 January 1982.

Nigel Williams is a member of the editorial staff of *Nature*.

types which release factors, from which hybridomas have been produced. Again such factors carry *I-J*-region determinants and have antigen-binding (or in the case of the second type TsF_2 , idiotype-binding) specificity. Preliminary experiments from immunoabsorbant columns were described which showed that the activity of TsF_2 could be reconstituted from equal amounts of eluates from an anti-idiotype column and anti-*I-J*-column following treatment of the factor under reducing conditions. This suggests that this factor in the series consists of two chains linked by a disulphide bond.

As T-cell hybridomas provide a monoclonal source of an increasing array of molecules it is crucial that their function is clearly established. Stressed by J. Miller, this point is underlined by a recent report by D. Capra (Pacífico & Capra *J. exp. Med.* **152**, 1289; 1980). He originally claimed (Clark & Capra *J. exp. Med.* **155**, 611; 1982) to have produced a hybridoma clone releasing an antigen-specific suppressor factor bearing *I*-region-encoded determinants. However the factor was not functionally tested and it now appears that the molecule can be recovered from lysates of other unrelated cells, although it is not yet known if this molecule carries *I*-region determinants.

The function of the T-cell molecules is one of the central questions facing immunologists; the other is that of their relationship to antibodies. The antigen-binding portion of an antibody molecule is encoded by a group of variable region genes. The rest of the molecule is encoded by constant region genes (those for the heavy chain determine the class of antibody). It is not yet clear whether the antigen-binding region of the T-cell molecules is encoded by antibody variable region genes. Some seem to bear variable region determinants while others do not.

Possible evidence for constant region determinants was described by E. Culbert (University College London). He has produced hybridomas from antigen-specific helper and suppressor T cells. Rabbit antisera raised against these factors appear to be able to discriminate between helper and suppressor hybridomas regardless of antigen specificity. Taniguchi has antisera that seem to detect a constant region determinant on the antigen-binding chain of his two-chain suppressor molecule. These seem to be encoded by genes close to the heavy chain constant region genes of antibody, as the antisera, raised in antibody heavy chain congenic mice, can detect determinants selectively expressed on T cells.

It is clear that the application of hybridoma technology is helping to provide an immortalized source of T-cell molecules. Although several different antigen-specific molecules have now been produced by these means, their precise relationship to antibodies remains tantalizingly obscure. □

How cells in distress use SOS

from S.G. Sedgwick and G.T. Yarranton

TREATMENT of eukaryotic and prokaryotic cells with DNA damaging agents induces a diverse and somewhat bewildering variety of cellular responses. One interpretation is that cells in general coordinately control operons which are expressed only in response to DNA damage. A model system of DNA damage-induced gene expression is the SOS response of *Escherichia coli*. This response entails de-repression of at least 12 different operons. The response alters DNA repair capability, mutagenesis, restriction modification and cell division control, and leads to expression of several different *din* genes, identified only by their inducibility by DNA damage. In addition, certain prophages, such as λ , are de-repressed and enter a cycle of lytic growth. It has been supposed that the phages have evolved to take advantage of the SOS response so that they can abandon a host cell whose viability is uncertain.

Although the genes which are switched on participate in several metabolically distinct pathways, they appear to be part of a single regulatory unit, the SOS regulon, whose expression is controlled by the *lexA* and *recA* genes. The idea that *lexA* protein is a multisite repressor switching off transcription of the *recA* and several other genes, including its own, has recently gained direct support.

Little *et al.*² and Brent and Ptashne³ have purified *lexA* protein and examined its binding to purified control sequences of the *lexA* and *recA* genes. Both DNAs contained 20 base pair palindromic sequences, or SOS boxes, which were protected by *lexA* protein against nuclease and methylation treatments. The *recA* gene has a single box, whilst the *lexA* gene has a tandem pair. Significantly, all three binding sites show considerable homology with one another^{2,4} and with sequences preceding two other genes, *uvrA* and *uvrB*^{5,6A}, which are also part of the SOS regulon. Repressor activity *in vitro* was demonstrated directly by specific inhibition of transcription from both *lexA* and *recA* promoters by purified *lexA* protein. Further evidence of multigenic repression by *lexA* protein was provided by analogous experiments in which *lexA* protein blocked *in vitro* transcription from cloned *uvrB*^{6A} and *dinA* and *dinB* genes⁷. Thus, during normal growth, the genes of the SOS regulon would be repressed by *lexA* protein which would be maintained at a constant level by the autoregulatory repression which *lexA* protein exerts on its own synthesis.

Predictions that induction of the SOS

regulon is caused by proteolytic inactivation of *lexA* protein by *recA* protein have also been confirmed. Little *et al.*² showed *in vitro* that homogeneous *recA* protein cleaves purified *lexA* protein into two fragments that cannot repress transcription from the *recA* promoter. They used a mutant form of *recA* protein that has stronger than normal proteolytic activity, but similar proteolytic action by wild-type *recA* protein is thought to be activated following damaging treatments by single-stranded DNA or DNA degradation products. The proteolysis of *lexA* protein therefore closely resembles the λ repressor cleavage reaction described by J.W. Roberts and his colleagues⁸. Indeed, the cleavage site is in a region with a similar composition to that of λ repressor⁹ and may thus provide a molecular example of convergent evolution — λ repressor has evolved to mimic *lexA* protein as a substrate of proteolysis by *recA* protein.

Clues to how finer regulation of the SOS response occurs are also appearing. First, *recA* cleaves *lexA* protein at least ten times more efficiently than it does λ repressor. This may favour survival of λ by ensuring that lysogenic induction does not occur until the levels of DNA damage exceed the capacity of the inducible repair systems⁹. Second, the *recA* SOS box binds *lexA* protein about ten times more strongly than do the pair of boxes preceding the *lexA* gene³. Thus, various SOS operons with different variations of the SOS box sequence may be derepressed after different amounts of *lexA* protein have been inactivated. The reason why there are two SOS boxes at the *lexA* locus is not clear, though Brent and Ptashne suggest that their lower affinity would permit *de novo* *lexA* protein synthesis during induction. As a result, the period in which *lexA* levels fall might be extended, permitting finer discrimination for switching on part, but not all, of the regulon. Lower repressor affinity of the *lexA* operator might be expected on theoretical grounds since the gene must presumably synthesize sufficient product to ensure repression of the rest of the regulon before it switches off its own expression.

Third, the relative position of SOS promoter and operator sequences vary^{2,3}. However, the importance of this feature is unclear and the relative binding constants of promoters at different sites must be determined before the complete kinetic analysis of gene expression during induction can be made.

A second refinement of the basic SOS model must be introduced to account for at least two genes which can be regulated independently, or as part of the SOS regulon. The *uvrB* gene, which codes for a

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subunit of the UV endonuclease, was found by Fogliano and Schendel¹⁰ to be under *lexA* control. Subsequent sequence analysis of the *uvrB* control region^{5,6A} revealed adjacent non-overlapping promoter sequences. The P2 promoter, furthest from the initiation codon, spans an SOS box⁵ protected from *in vitro* nuclease digestion by *lexA* protein, suggesting that this is the site where *lexA* repression occurs. Control of expression from P1 is as yet unresolved, although van den Berg *et al.*¹¹ suggest that the *uvrC* protein may be involved. Interestingly, Sancar *et al.*¹¹ have identified and purified the *uvrC* protein and shown that it binds strongly to DNA. The third promoter, P3, located 341 base pairs before the structural gene, produces an *in vitro* transcript terminating in the region of *lexA* binding site. It is unclear whether P3 has a role in *in vivo uvrB* gene expression^{6A}. Identification of a second gene, *himA*, with dual control has recently been described by Miller *et al.*¹². The *himA* gene product is needed for site-specific recombination, best studied by integration of phage λ DNA into the *E. coli* chromosome. λ integration is accomplished by the combined action of λ *int* protein and host integration factor. *himA*'s function is to encode one subunit of the host integration factor, the other subunit is probably specified by the *himD* gene. *himA* was found to resemble other genes in the SOS regulon in being induced by UV and mitomycin C provided the *recA* and *lexA* genes were functioning normally. However, *himA* and *himD* are also subject to an alternative control mechanism, since both genes are expressed at high levels when either one of their gene products is inactive. This indicates that *himA* and *himD* may jointly repress their own syntheses as well as forming host integration factor. Until the controlling regions of these genes have been sequenced, it is uncertain whether or not *lexA*, *himA* and *himD* proteins act at the same or different sites, and whether *himD* is also regulated by *lexA*.

Remarkably, the role of *himA* in lysogenization is not limited to providing a subunit of the host integration factor. Earlier work by Miller¹³ showed that *himA*

was also needed for efficient transcription from the λ promoters P_E and P_I , which direct synthesis of λ repressor and integrase. Miller suggested that *himA* possibly repressed the bacterial *hfl* gene whose product decreases activation of P_E and P_I by λ CI protein. Thus, *himA* also aids lysogenization by boosting synthesis of λ repressor and integrase. *Int* protein and host integration factor are also required for prophage excision during lysogenic induction, showing again how λ exploits

the SOS system to leave a host which might be dying.

The existence of at least two genes with dual regulatory mechanisms raises the possibility of a general scheme where many components of the SOS regulon may be capable of independent expression for 'routine' metabolism. Superimposition of a second SOS regulatory system would provide a means of coordinating diverse and unrelated metabolic pathways to aid cellular survival. □

A Soviet view of the venusian surface

from Lionel Wilson

A highlight of this year's Lunar and Planetary Science Conference* was the session devoted to Venus, at the close of which Soviet scientists presented photographic panoramas and chemical analyses of the Venus surface obtained by the recent soft-landers Venera 13 and 14. The resolution of the new images is far better than those from earlier Soviet probes and the chemical data provide the first opportunity to make direct inferences about the internal geochemistry of Venus.

Studies of the Solar System over the last decade have shown that the other silicate planets — Mercury, Venus and Mars — display a variety of surface features produced by processes identifiable on Earth; but no planet yet examined has a crust dominated by the kinds of large-scale, plate tectonic processes which typify the Earth's outer layers. Until recently, however, the surface of Venus, the only planet truly comparable with the Earth in terms of size and mass, remained hidden beneath the dense, cloud-rich, CO₂ atmosphere. In 1975, the soft-landing Soviet spacecraft Venera 9 and 10 provided low-resolution pictures of two parts of the surface, showing boulder-strewn terrains; but, although there was evidence of vesicular rocks¹ these pictures only gave hints at the structures visible and the processes which might have formed them.

Attention switched to the large-scale structure of the crust of Venus when the US Pioneer Orbiter reached the planet late in 1978 and began to map the surface topography by radar. A consensus of opinion² held that Earth-style plate tectonic processes were at best poorly developed on Venus and that heat was released from the interior at a series of localized mantle hot-spots above which active volcanoes opinion, held that Earth-style plate tectonic crustal uplifts, the most prominent being the Beta Regio region. The Pioneer measurements, coupled with Earth-based radar data on surface roughness and dielectric constant, showed that the southern part of Beta Regio was one of the

few areas where fresh, dense rocks, possibly recently erupted basaltic volcanics, were exposed at the surface.

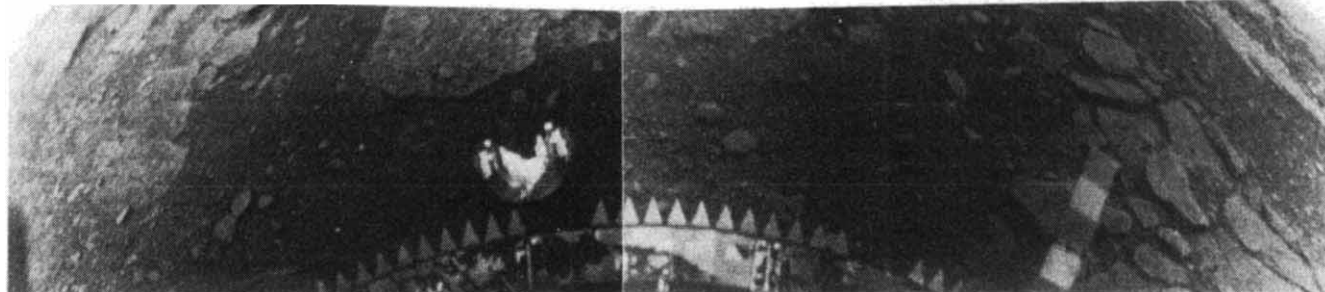
Not all those studying Venus are reconciled to the absence of plate tectonic processes, however, and the debate continued at the Houston meeting. On the one hand, H. Spetzler and K.A. Goettel argued for a weak crust^{3,4} and J.L. Warner provided a mechanism⁵ for recycling the crust via the subsidence of interlayered volcanic rocks and aeolian sediments; in contrast, S. Solomon reviewed the reasons⁶ why we should not yet rule out any of the possible styles of tectonism proposed for Venus. In an attempt to sidestep the issue of tectonic regimes, L. Wilson and J.B. Garvin^{7,8} showed that, irrespective of magma chemistry, eruptions on Venus were likely to produce lava flows longer than those on Earth. Because of the high surface pressure on Venus (55–90 bars), explosive eruptions which produce dispersed ash layers could only occur if the volatile contents of erupting magmas were high by terrestrial standards, exceeding about three weight per cent.

The new Soviet results bear on all aspects of the topics outlined above. High-resolution images (showing millimetre-sized particles in the foreground) were obtained through red, green and blue filters at both landing sites and colour composites produced. In each case, the sky appeared to have a yellow hue while exposed rock surfaces were a yellowish orange and fine-grained materials showed a greenish tint. A drilling device obtained samples (thought to be mainly low-strength soils) from the vicinity of each lander and transferred them into an X-ray fluorimeter

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*The 13th Lunar and Planetary Science Conference was held at the Lyndon B. Johnson Space Center, Houston, Texas, on 15–19 March 1982.

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system for major element chemical analysis (Table 1).

Table 1 Oxide analyses of Venus surface rock in weight per cent

Oxide	Venera 13 site	Venera 14 site
SiO ₂	45	49
Al ₂ O ₃	16	18
MgO	10	8
FeO	9	9
CaO	7	10
K ₂ O	4	0.2
TiO ₂	1.5	1.2
MnO	0.2	0.16

At the Venera 13 site, on rolling uplands high on the flanks of Beta Regio, extensive coherent strata are seen, together with isolated rocks showing some evidence of erosion. The composition of the rock sample is close to that typical of terrestrial

alkali basalts found in continental rift zones — a striking result when one recalls the proposed origin of Beta Regio as a rifted uplift over a hot-spot. Alkali basalts on Earth are associated with magma source regions rich in CO₂ and the existence of such rocks on Venus lends support to the idea that CO₂-driven explosive volcanism may occur there despite the high surface pressure.

The surface at the Venera 14 landing site, in a lowland area south-east of Beta Regio, is dominated by extensive horizontal strata, 10–100 mm thick, which have the appearance of lithified sediments in the process of being eroded. The composition, however, is again very close to that of a terrestrial primary volcanic product, in this case an ocean-floor tholeiitic basalt. It is tempting to reconcile the composition with the sedimentary character of the rock by assuming that it is some kind of pyroclastic material, but then the problem of finding

sufficient volatiles to drive the eruption reappears. We should not jump to the conclusion that the tholeiitic nature of the Venera 14 rock necessarily implies that it was produced in a plate tectonic environment, as such a rock type would be on Earth. Indeed, too much must not be inferred about the interior of Venus from just two rock analyses; but it is ironic that each of the two analyses available, interpreted naively, supports one of the two main theories of Venus crustal structure. □

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Punctuationism and Darwinism reconciled?

The Lake Turkana mollusc sequence

On 8 October, 1981 P G Williamson¹ presented an extraordinarily “complete page in the history of evolution” in a very detailed sequence of fossil molluscs from the the eastern Turkana basin in East Africa. All of 13 lineages, including ten which could be followed for 4–5 million years, showed a pattern of evolution conforming to the ‘punctuated equilibrium’ model: change was concentrated in short (5,000–50,000 years) speciation events, between long periods (3–5 million years) of morphological stasis. How is such a pattern to be explained?

In the same issue, Jones² pointed out that the rapidity with which change occurred is certainly no greater than can be brought about by conventional Darwinian selection. The real problem, as Williamson³ and others⁴ have made clear, is to explain the very long periods of stasis. Williamson favours the view, originally put forward by Eldredge and Gould⁵, that stasis is primarily the result of “developmental constraint”, which makes certain kinds of developmental change difficult or impossible, and that speciation must necessarily involve the dismantling of these mechanisms. He sees evidence of this process in the periods of extreme developmental instability (recorded as an increase in phenotypic variance) that accompany speciation events in his mollusc sequences.

A contrasting view is that stasis may be explained by stabilizing selection — that is selection favouring the typical members of a population rather than the extremes. If this explanation is correct, then as Maynard Smith points out in a new book (*Evolution Now**) what we need is “a theory which says something about selection, and hence about the environment. Since the major component of the environment of most species consists of other species in the ecosystem, it follows that we need a theory of ecosystems in which the component species are evolving by natural selection.”

Williamson’s articles have stimulated a great deal of comment, a small part of which appears below. Questions are raised both as to the nature of the pattern recorded in the fossil lineages — whether or not the bursts of rapid change are truly genetic in origin — and its implications. The notion of developmental constraint is debated and more general points, such as whether the ‘punctuated equilibrium’ pattern could be brought about by varying rates of environmental change, or whether it could be an artefact of sampling time scales, are also examined.

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*See foot note on p.601.

Questions concerning speciation

from Ernst Mayr

THE pattern of evolution in the mollusc lineages reported by Williamson seems to be in excellent agreement with the theory of punctuated equilibria, which is based on the well-known paleontological observation that new species usually enter the fossil record abruptly (in geological time) and postulates that they persist subsequently with little significant change until extinction. The rapid change under the stress conditions of an evaporating Lake Turkana is, as Williamson points out, less of a puzzle than the millions of years of stasis.

Williamson refers to my observation that many currently living species show phenotypic uniformity even though their range extends over areas of considerable ecological and climatic diversity. This observation strengthens the thesis that it is not stabilizing selection of a set of essentially additive genes that is responsible for the stasis but rather the strength of the "internal balance" (Mather), "genetic homeostasis" (Lerner), "cohesion of the genotype" (Mayr). These authors postulate that the genotype as a whole is a finely balanced system, in which appropriate feedback mechanisms maintain morphological stability by compensating for whatever genetic changes occur through time at individual loci.

Williamson has presented a plausible explanation of his findings and it might well be the correct one. However, in his short communication, he has not refuted conceivable alternate theories nor has he provided answers to some disturbing questions that might arise in the mind of the reader.

First, Williamson states that the changes in 12 lines of sexually reproducing molluscs are paralleled exactly by events in the lineage of the asexually reproducing species *Melanoides tuberculatus*. Since an asexual phyletic lineage consists of hundreds, if not many thousands, of independent clones, how does Williamson explain that all of them experience a parallel history of equivalent genetic changes? This concordant phenotypic change of the scores of asexual clones suggests that the changes are not genetic at all, but merely modifications of the phenotype (see also the letter below from A.J. Boucot).

Second, does the variability of the asexual (*Melanoides*) lineage, consisting of numerous independent clones, differ from those of the sexual lineages?

Third, Williamson states that the new 'species' become suddenly extinct when the

widespread parental species invade the Turkana basin. There are four sets of possibilities for the behaviour of parental and daughter "species" at the time of the establishment of sympatry: (a) Conditions in the Turkana basin, before the reinvasion by the widespread parental stocks, had deteriorated to such an extent that the new "species" had all become extinct already; (b) They had become reproductively isolated; in that case it should be possible to find them sympatrically in zones of contact; (c) They had not yet developed reproductive isolation and it should be possible to find hybrid populations with greatly increased variability; (d) The observed morphological differences of mother and daughter 'species' had no genetic basis but were merely phenotypic responses to different environmental conditions; in that case a sudden and total change of the phenotype should accompany the replacement of the lake waters. A fine-grained analysis of the populations occurring during the crucial period of reversion to standard conditions should permit an answer to these questions.

Finally, one would also like to know more about one set of puzzling speciations. In six of the 13 lineages a second species

apparently originated in the lower Koobi Fora Formation (*Bellomya unicolor*, *Cleopatra ferruginea*, *Melanoides tuberculatus*, *Caelatura bakeri*, *Mutela nilotica*, and *Eupera ferruginea*). All six of these daughter species seem to become extinct before the end of the lower Koobi Fora (see Fig. 4). Do these six new daughter species coexist sympatrically with the parent stock without producing intermediates? Does Williamson believe that they originated in some peripheral isolate (a temporarily isolated lagoon?) and were able, after a rise in lake level, to invade the range of the parent stock? Several other questions are obvious, but one can not even begin to answer them until Williamson produces more factual evidence about these six postulated speciation events.

Williamson states that, on the whole, his findings are consistent with my theory of peripatric speciation in peripheral founder populations, with two drastic exceptions: The asexual *Melanoides* behaves like the sexual lineages, a fact not explained by Mayr's postulate of genetic revolutions, and the size of the shell deposits indicates that the 13 (19) lineages never truly seem to have passed through a narrow population bottleneck, as demanded by the theory of peripatric speciation. It is impossible to resolve this conflict — Williamson himself does not try to do it — until much better factual evidence for some of the events in the sequence of these mollusc faunas is presented. □

Ecophenotypic or genotypic?

from Arthur J. Boucot

WILLIAMSON's brief comment (p. 441) that the presumably derivative morphologies, all arrayed in two, well-dated, very discrete horizons and one more diffuse horizon, cannot be considered to be ecophenotypic morphologies of the unchanging, long-ranging forms — many of which persist to the present — needs discussion. It is essential that in an area where lake waters of widely different composition are known to have occurred in the past, as well as the present, that the possibility of ecophenotypic, as contrasted with a genetic, change be dismissed only when adequate data has accumulated. One would have hoped that Williamson would have provided us with observational data, based on those species that persist to the present, showing that under conditions of varied lake water composition (both concentration and composition) there are no ecophenotypic effects.

If such data are unavailable, Williamson could have cultured at least some of the living species under conditions of salinity and water composition that might have

occurred in parts of the Turkana Basin region during the time interval studied. Without such data one must be forgiven for still considering that the possibility of ecophenotypic change is fully as rational as that of speciation. Few neontologists would adhere to the tenet that distinctly different skeletal morphologies within a genus invariably indicate different genotypes. Paleontologists normally assign distinctly different morphologies within a genus to different species, lacking evidence for a well documented morphologic gradient of the ecophenotypic type, only because they commonly deal with extinct genera; not because they feel that the procedure is invariably defensible.

Finally, Williamson really should have provided us with a satisfying explanation of why speciation events should have occurred simultaneously, at two distinct

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and one diffuse horizon, within a variety of unrelated molluscs. This certainly is a strange coincidence from an evolutionary point of view, although entirely plausible from the ecophenotypic point of view if a major change in lake water chemistry and concentration affecting an arm of a large

lake was involved. The long stratigraphic range of those stocks still represented today makes it clear that they were present in the region throughout the entire time interval, whereas all of the morphologies derived from them during the brief intervals of morphologic change

lasted a very short time — this situation is entirely consistent with either local speciation under unknown physical conditions, or under locally isolated conditions involving different enough conditions to produce distinctive ecophenotypes. □

Morphological stasis and developmental constraint: no problem for Neo-Darwinism

from Brian Charlesworth and Russell Lande

THE critical issue stemming from Williamson's papers is the explanation of stasis and increased variability associated with the episodes of change. The view he holds, that stasis is due to developmental constraints², is equivalent to saying that the characters concerned lack genetic variability, so that selection is ineffective. But there is evidence for substantial levels of heritable variability in most morphological characters that have been studied^{1,4}, including snail shell traits^{5,6}.

Stasis cannot, therefore, be explained by developmental constraints; either the characters are selectively neutral and population sizes are too large for genetic drift to be effective, or natural selection acts towards an intermediate optimum phenotype. Direct evidence for such stabilising selection on shell characters in snails was provided by the early biometricians^{7,8}, and similar data is available from many other traits⁹. Williamson is also incorrect in implying that stabilising selection has only recently been invoked by neo-Darwinists as an explanation of stasis; for example, Simpson (ref. 10, pp148–150 and 327–355) discusses this question at length.

The observation of increased variability

at times of rapid change could have several explanations not discussed by Williamson. He neglects the possibility that it could be caused by a direct developmental response to increased heterogeneity of the habitat, or by the effects of temporal fluctuations during individual lifetimes, at periods when the environment is changing. Furthermore, since fossils classed as belonging to one geological horizon almost certainly span many generations, rapid evolutionary change over the period of sampling could generate increased variation. For these reasons, we are not convinced that the increased variation is a genuine evolutionary phenomenon. But even if this is granted, there would be no difficulty for neo-Darwinism. Selection experiments have demonstrated that stabilising selection can enhance developmental stability, or zones of

'canalisation', around an optimum phenotype by altering the patterns of genetic and non-genetic variation^{11–13}. Relaxation of stabilising selection¹⁴, and/or directional selection away from a zone of canalisation^{15–17}, would then be predicted to produce an increase in phenotypic variability, until the population is re canalised by stabilising selection around a new optimum phenotype¹⁸ (Kirkpatrick, *Am.Nat.*, in press). However, many characters under weak stabilising natural selection are not appreciably canalised in their development, and can be artificially selected to change a great deal without substantially increasing their variability⁴. The exact temporal patterns of variability expected thus depend on factors whose relative weights are difficult to assess for fossil material. □

Are 'punctuations' artefacts of time-scales?

from Lev R. Ginzburg and Jay D. Rost

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WE would like to give a brief explanation of why the 'punctuated' pattern of evolutionary change seen in Williamson's and other, less well documented, findings may well be an artefact of the sampling time scales. A more detailed argument is presented elsewhere¹.

Consider the following imaginary example: A population of *E. coli* is cultivated in a chemostat under a fixed environment for a number of years. Assume that we sample cells from the culture with different time intervals to discover whether the culture has evolved or not with respect to a quantitative trait. If we do this, bi-weekly, for instance, we will find most of the time that nothing has happened. Occasionally we will find changes that appear as punctuational, since the replacement time in a chemostat is typically much shorter than two weeks. Now, if we make our observations hourly, the details of the replacement process will be obvious and the evolutionary change will appear gradual. If we make only yearly obser-

vations, the process may look gradual for a different reason; we would average a number of changes during the year into one yearly change.

If we repeat the experiment with a number of isolated chemostats, and carry out bi-weekly sampling for a period of a few months, we expect most of the populations to show no change, but some of them will evolve away from the majority. This may look like a punctuative "speciation event." Too fine or too coarse a time scale will always lead to a more "gradual" picture, whereas at some intermediate scale, the process will appear as "punctuational." In the work of Williamson the mean time scale resolution is between 30,000 and 35,000 years. (The value is the sum of the time spans of segments 2, 3, 4 and 5, about 2.5 Myr,

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divided by 79 sample points). If a typical replacement time is significantly less (selective advantages of 10^{-4} – 10^{-5} per year), our hypothesis may well be correct. Note that other well-documented studies claiming support for gradualism appear to have scale resolutions significantly greater than this. These researchers may be sampling at too coarse a time scale to detect 'punctuational' events (See Table below).

Table

	Studies in support of gradualism	Time-scale resolution (total time span/total no. of samples)(yr)	Organism
a	Cronin <i>et al.</i> ²	625,000	Hominids
b	Ozawa ³	440,000	Foraminiferans
c	Gingerich ⁴	90,300	Condylarths
d	Kellogg ⁵	74,700	Radiolarians

a, 625,000 = 2.5 Myr time span divided by four sample points (measuring cranial capacity). Includes specimens of *Australopithecus africanus*, *Homo habilis*, *Homo erectus* and *Homo sapiens*. b, 440,000 = 15 Myr time span divided by 34 sample points. c, 90,300 = 3.07 Myr time span divided by 34 sample points; *Hyopsodus*. d, 74,700 = 2.54 Myr time span divided by 34 sample points. All total time spans were estimated from author's graphs.

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Punctuated equilibria and punctuated environments

from D.W. Lindsay

EVOLUTION is not an event which occurs without external agency. A selective pressure is necessary for evolution to occur and must be an important determinant of the rate of evolution. Where such rates appear to vary, as Williamson's study shows, surely the first aspect of the problem to receive attention should be the selective pressures involved. In a stable lacustrine environment, as seems to have existed between the bouts of 'speciation' described by Williamson, selective pressures may have been low or minimal so that a lack of change in a few correlated characters seems rather unsurprising. Equally unsurprising are sudden series of changes coinciding with 'major lacustrine regressions'. These would clearly introduce marked selective pressures and a consequent flurry of genotypic and phenotypic experimentation until adaptation had been attained, or not,

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and the environment stabilised. Thus, observations of stasis interspersed with rapid change may simply reflect changes in the direction and intensity of the selective pressures which are implied by the stratigraphic record. Generally, we ought to consider a punctuated environment in thoughts on punctuated evolution. It is a sad fact of paleontology that the conditions of stable, prolonged sedimentation which favour preservation may lead to low rates of evolution, while periods of environmental hiatus which would have accelerated the process may often be lost in the unconformities. □

Williamson replies:

In answer to Mayr's first point: It is surely unnecessary to postulate that *all* the clones of *Melanoides* present in the Turkana Basin underwent parallel genetic changes during the episodes of morphological transformation at the Suregei and Guomde levels. It seems more probable that one, or a few, such clones made an appropriate evolutionary response to the relevant selective pressures, and that, as these few clones evolved, they proceeded to outcompete other 'conspecific' clones. Such interclonal competition is well documented¹, and Mayr himself has suggested competitive elimination of inferior clones as the probable mechanism whereby discontinuities between closely related asexual 'species' arise². I address Mayr's further suggestion (that the morphological transformations documented in the Turkana sequence are purely ecophenotypic reactions) below.

In response to Mayr's second point: The variability (c.v.) of all measured traits in populations of the asexual taxon *Melanoides tuberculata* are quite comparable to the variabilities documented in typical populations of the gonochoristic gastropod taxa³.

Mayr's third point: The details surrounding the sudden reinvasion of parental stocks into the basin above the Suregei Tuff level speciation event, synchronous with a major lacustrine transgression, are at present poorly understood. Some 2-3 metres of fossil-barren sediment separates the last of the novel Suregei level forms from the first of the returning ancestral stocks. No hybrids, sympatric faunas or intermediates are currently known. It is not therefore possible to distinguish between Mayr's options (a), (b) and (c). But as discussed below, Mayr's option (d) appears untenable.

Mayr is correct in noting a set of speciation events in certain lineages in the Lower Member of the Koobi Fora Forma-

tion. These new forms appear suddenly in the record and coexist with their ancestral stocks; the event is therefore a modest adaptive radiation typical of that seen in many modern rift lakes. All these new endemics become extinct by the top of the Lower Member, due to a climatically induced regression — and consequent increase in alkalinity — well documented in the uppermost part of the Lower Member. It is indeed likely, that these new endemics arose in peripheral isolates of the large Lower Member lake, perhaps in the manner suggested by Hubendick⁴.

With reference to Mayr's last paragraph: I believe that it is indeed possible to resolve the conflict between Mayr's model of peripatric speciation and my observations in the Turkana Basin. I hope to address this topic in a forthcoming paper.

Boucot — and also Mayr — suggest that the novel forms I document as arising at the Suregei, Guomde, and Lower Member levels may simply be ecophenotypic 'reactive forms' of the 'ancestral' lineages. Several factors strongly indicate that this is not the case: (1) the novel forms arising in the Lower Member *coexist* with their presumed ancestral forms, and occur with them in the same 'life-assemblages'. The Lower Member novelties at least can not be simple ecophenotypic variants⁵. (2) The principle stem lineages in the Turkana Basin sequence are still extant and widely distributed in Africa, but even the most extreme modern environments in which these lineages occur at present produce a simple dwarfing of characteristic morphology (or *in extremis*, extinction of local populations), they never produce the striking reorganization of phenotype documented in the Turkana Basin sequence⁵. (3) The morphological transformations documented at the Suregei Tuff level are invariably unidirectional in character space, and required some 10^3 – 10^4 generations to accomplish⁶. This is hardly the nature or time-scale of a conventional ecophenotypic response⁵. The *magnitude* of phenotypic change during the Suregei and Guomde Level episodes is striking. In the bivalves, these changes involve a radical restructuring of shell form and hinge dentition, and major changes in the disposition of the muscle scars. The magnitude of the changes documented in both bivalves and gastropods is generally far greater than that observed in the ecophenotypic transformations of even the most plastic of modern African freshwater molluscs³. I do not therefore believe that the profound morphological transformations documented in the Turkana Basin sequence can be simply 'written off' as an ecophenotypic response.

Boucot expresses surprise that *all* lineages at the Suregei and Guomde levels speciate. But it has long been acknowledged that two major 'triggers' for speciation events within peripheral isolates are (1) isolation *per se* and (2)

environmental stress⁷. The Suregei and Guomde Level speciation events occur at times of lacustrine regression. The various molluscan lineages have similar vagilities, and occupy similar environments. All would therefore experience both isolation and stress during a major regressive phase. A broadly synchronous evolutionary response in all lineages is therefore reasonable.

Charlesworth and Lande are incorrect in assuming that evidence for substantial heritable variability in morphological characters implies that morphological stasis cannot be attributed primarily to developmental constraint. The question is not so much whether variation exists for selection to work on, but rather what, if anything, selection can actually *make* of this variation in nature. Developmental constraint may well block long-term directional morphological trends in large populations, even though the genetic potential for such evolutionary transformation exists. This is at least one tenable explanation for the fact that, in the fossil record, such long-term morphological trends are rare, or absent, in continuous phyletic sequences.

Morphological stasis is not, therefore, a reflection of selective neutrality of phenotypes or simple stabilizing selection. The idea that most phenotypes are selectively neutral is not widely held, nor supported by Charlesworth and Lande's own references⁸. Neither is simple stabilizing selection an adequate explanation for long term morphological stasis. Many species — perhaps most — exhibit stasis for millions of years. The idea that such species experience an unchanged selection regime for these immense spans of geological time seems inherently unlikely. Conventional neo-Darwinists have similarly recognised that simple stabilizing selection is an inadequate explanation for the analogous situation of range-wide phenotypic coherence in modern species; they therefore invoke developmental constraints of one form or another to explain this coherence⁹. In asserting such constraints to be the principal element in long-term *temporal* stasis, punctuationalists are following the lead of conventional neo-Darwinian students of *geographic* variation.

Certain neo-Darwinists have recognised the primacy of developmental constraint in maintaining evolutionary stasis. For example Rendel¹⁰ states that "when a canalised character is to be changed . . . the unfitness introduced when the fine adjustment between developmental processes is destroyed will always be counteracting directional selection . . . directional selection must always introduce unfitness. This antagonism between fitness and directional selection will have to be taken into account in interpreting . . . long [term] stability. . ."

Charlesworth and Lande suggest that the greatly enhanced phenotypic variability I

document during periods of morphological transformation in the Turkana sequence simply represents a direct developmental response to increased heterogeneity of the environment. No evidence of any similar response is known from any modern population of the lineages concerned, even in the extremely wide range of environments many of them currently occupy; it hardly seems necessary to invoke them in this case. They then suggest that this variability might be due to 'mixing' of more- or -less evolved and stratigraphically adjacent populations. But as I point out⁵, the faunal units concerned seem to be undisturbed life assemblages with no evidence of reworking, and much of the variability derives from the presence of phenodeviants bearing as little relationship to the derived species as they do to the ancestral form. This does not suggest that the increased variability I document is due to the mixing of the products of a conventional neo-Darwinist evolutionary 'march of means'. It suggests pronounced developmental instability in the transitional forms. Charlesworth and Lande finally concede that this increased variability may represent developmental instability resulting from strong directional selection away from a zone of canalization — which is, after all, the explanation I offered in my original paper.

Ginzburg and Rost are correct in considering the gradualist and punctuational models to be, in part at least, alternate hypothesis about the *distribution* of rates of evolutionary change. But the critical issue in this context is the extent to which observed rates of change in a given interval can be extrapolated up into rates of change occurring in any longer time interval.

If Ginzburg and Rost's bacteria were evolving according to the gradualist model, the amount of change observed over any given sample interval could be extrapolated up to account for the net change occurring over any larger sample interval. If the bacteria were evolving according to the punctuational model, the amount of change observed over any given sample interval could not be extrapolated up into the net amount of change observed over a much larger interval. In the latter case, if a given sample interval happened to include a 'speciation event', estimated rates of net change over a larger sample interval would be much too high (long periods of stasis would occupy most of a longer time period). If, more likely, one happened to sample a period of stasis, extrapolated rates of change for a larger sample interval would be much too low, that is effectively zero.

The sampling time scale is therefore irrelevant to the punctuationalist-gradualist controversy. In any case, much more than simple differences in rate of evolutionary

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100 YEARS AGO

On Tuesday evening, April 11, the public thoroughfare stretching between Hatton Garden and the Old Bailey was lighted for the first time by the electric light. The novelty of the installation was the fact that the incandescent system had been adopted in preference to the arc system. The Holborn street lamps each contain two of Edison's bulbs suspended from a cross bar running through the top of the lantern. The light is of a golden tinge like gas, but much purer, brighter, and steadier. The lamps were switched on and off with the greatest ease, and altogether the experiment was a complete success.

Mount Etna has again been in an active condition. An eruption and a rain of ashes (rampilli) has quite recently alarmed the neighbouring inhabitants.

from *Nature* 25, 564-5; April 13, 1882.

change are involved in the differences between the punctuated and gradualist models: for example, the size of the populations in which significant evolutionary changes is thought to occur is also a critical distinction between these two models¹¹.

Lindsay suggests that selection pressure is a *sine qua non* of evolutionary change. He ignores such staples of conventional neo-Darwinism as founder effect, genetic drift, mutation pressure and the appearance of 'super fecund' mutants, all of which phenomena are thought to effect evolutionary change irrespective of — or even in opposition to — selection pressure. But Lindsay ignores these, and suggests that the 'variation in rates' I observe in the Turkana section are the simple consequence of variations in the Suregei and Guomde Level although speciation events occupy only 5×10^3 – 5×10^4 yrs represented by the Turkana sequence. In other words, according to Lindsay, for only 1 per cent or 0.1 per cent of the time represented by the Turkana Basin sequence were selection pressures adequate to elicit any evolutionary response in any lineage. For the remaining 99 per cent or 99.9 per cent of the time, selection pressures were 'so low or minimal' — for 5 Myr — that no significant change occurred whatsoever in any lineage. Lindsay describes this as unsurprising. I find it incredible! In fact, even the most conventional of neo-Darwinists would agree that rates of evolutionary change are controlled by many more factors than simple fluctuation in selection pressures.

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REVIEW ARTICLE

The role of protein phosphorylation in neural and hormonal control of cellular activity

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Protein phosphorylation is now recognized to be the major general mechanism by which intracellular events in mammalian tissues are controlled by external physiological stimuli. However, only recently has the idea that different cellular functions are controlled by common protein kinases and protein phosphatases started to gain widespread acceptance. Thus there is an integrated network of regulatory pathways, mediated by phosphorylation-dephosphorylation, that allows diverse cellular events to be coordinated by neural and hormonal stimuli. The evidence that supports this concept is reviewed, with emphasis on the role of protein phosphorylation in enzyme regulation.

THE presence of phosphorus in proteins has been known for almost 100 years, but its importance has only been realized since the discovery of enzyme regulation by reversible phosphorylation. The current excitement stems from the work of Krebs, Fischer and Larner over the period 1955–70, when they discovered that the neural and hormonal control of glycogen metabolism in skeletal muscle was mediated by changes in the phosphorylation state of glycogen phosphorylase¹, phosphorylase kinase² and glycogen synthase³ (Fig. 1).

These three enzymes remained the only examples of this phenomenon until the late 1960s, but the situation changed rapidly following the discovery of cyclic AMP-dependent protein kinase (cAMP-PrK)⁴. The past 10 years have seen an extraordinary and still accelerating growth in this area. About 35 enzymes and countless other proteins are now known to be regulated in this manner, and protein phosphorylation is clearly the major general mechanism by which intracellular events respond to external physiological stimuli⁵.

The first part of this review will summarize current knowledge of the control of glycogen metabolism, as this system is understood in the greatest molecular detail and continues to act as a model with which others are compared. The second part will present the increasing body of evidence that implicates many of the proteins involved in the control of glycogen metabolism in the regulation of other cellular activities.

Neural and hormonal control of glycogen metabolism in mammalian skeletal muscle

Glycogen is a major source of energy for muscle contraction. Its breakdown and synthesis are regulated by the contractile state of the tissue as well as by the hormones adrenaline and insulin, through changes in the phosphorylation state of glycogen phosphorylase and glycogen synthase (Fig. 1). These interconversion reactions are now known to involve five different protein kinases, four protein phosphatases, and regulatory proteins such as calmodulin, troponin-C, inhibitor-1 and inhibitor-2. Some of these proteins allow glycogen metabolism to respond to the second messengers Ca^{2+} and cyclic AMP (Fig. 1). The role of other proteins is less clear, although they may be involved in the action of insulin. Recent advances in understanding the molecular mechanisms underlying these reactions are summarized below.

Neural control via calcium ions: When muscle is stimulated electrically, calcium ions released from the sarcoplasmic

reticulum not only initiate muscle contraction but also activate phosphorylase kinase^{6,7}. As a result, the phosphorylation state of glycogen phosphorylase is increased^{8–10} and glycogenolysis is accelerated to provide the ATP required to sustain muscle contraction. Recently, phosphorylase kinase has been shown to phosphorylate glycogen synthase and to decrease its activity^{11–15}. Thus, the two opposing pathways of glycogenolysis and glycogen synthesis may be regulated in a synchronous manner during contraction.

Phosphorylase kinase is the key to both the neural and hormonal mechanisms for stimulating glycogenolysis, as it is not only dependent on Ca^{2+} , but is also a target for cAMP-PrK (Fig. 1). The enzyme has the subunit structure $(\alpha\beta\gamma\delta)_4$, where the α - and β -subunits are the components phosphorylated by cAMP-PrK^{16,17}, the γ -subunit seems to be the catalytic subunit¹⁸ and the δ -subunit is the Ca^{2+} -binding subunit^{19,20}.

The δ -subunit is identical to calmodulin^{19–22}, a protein originally discovered as an activator of a quite different enzyme but which is now implicated in the regulation of many Ca^{2+} -dependent enzymes (discussed below). Calmodulin binds four calcium ions per mol at micromolar concentrations²³, the affinity of the two Ca^{2+} -binding sites in the N-terminal portion being 10-fold higher than those in the C-terminal domain²⁴.

Phosphorylation by cAMP-PrK activates phosphorylase kinase ~15–20-fold at saturating Ca^{2+} concentrations and decreases the K_a for Ca^{2+} ~15-fold²⁵. Thus, phosphorylation not only enhances the activity of the γ -subunit, but may also allow activation to occur when fewer calcium ions are bound to calmodulin. This suggests a biological role for the different classes of Ca^{2+} -binding sites in calmodulin²⁶.

Phosphorylase kinase has been found to interact with a second molecule of calmodulin, termed the δ' -subunit, which only binds to the enzyme in the presence of Ca^{2+} where it interacts with the α - and β -subunits. In contrast, the integral molecule of calmodulin (the δ -subunit) remains complexed with the γ -subunit even in the absence of Ca^{2+} (refs 20, 27, 28). The δ' -subunit strongly activates the dephosphorylated form of phosphorylase kinase but has little or no effect on the phosphorylated form²⁵.

Calmodulin is closely related in structure and Ca^{2+} -binding properties to troponin-C²², the protein on the thin filaments of muscle that confers Ca^{2+} sensitivity to the contractile apparatus. Troponin-C, the troponin complex, and even artificial thin filaments of muscle, can substitute for the δ' -subunit, and several lines of evidence suggest that troponin-C rather than

Table 1 Classification of protein phosphatases in cellular regulation

Type	Protein phosphatase	Inhibited by I ₁ and I ₂	Specificity for phosphorylase kinase	Substrate specificity	Regulators
1	Protein phosphatase-1	Yes	β -subunit	Broad	I ₁ , I ₂ , GSK-3 + Mg ²⁺ -ATP
2	Protein phosphatase-2A	No	α -subunit	Broad	Unknown
2	Protein phosphatase-2B	No	α -subunit	Narrow	Ca ²⁺ -calmodulin
2	Protein phosphatase-2C	No	α -subunit	Broad	Mg ²⁺

Protein phosphatases-2B and -2C are completely dependent on Ca²⁺ and Mg²⁺, respectively, whereas protein phosphatases-1 and -2A are active in the absence of divalent cations. Protein phosphatases-1 and -2A have very similar substrate specificities. The specificity of protein phosphatase-2C is also broad, although it does not act on some phosphoproteins such as phosphorylase *a* (refs 52–55). Protein phosphatase-2B has a very restricted specificity, and of many phosphoproteins examined, only phosphorylase kinase (α -subunit), inhibitor-1 and myosin light chains have so far been found to serve as substrates^{69,70}. Abbreviations: I₁, I₂, inhibitor-1 and inhibitor-2; GSK-3, glycogen synthase kinase-3.

the δ -subunit may be the important activator *in vivo*^{25,26}. At physiological Ca²⁺ concentrations (1–3 μ M), the dephosphorylated form of phosphorylase kinase is activated ~20-fold by the troponin complex^{25,26}.

Figure 2 summarizes current ideas about the regulation of glycogen metabolism by Ca²⁺. Phosphorylase kinase is controlled by two related Ca²⁺-binding proteins, but their relative importance depends on the phosphorylation state of the enzyme. Troponin-C seems to be the dominant regulator of the dephosphorylated form, while calmodulin (the δ -subunit) determines the Ca²⁺ sensitivity of the phosphorylated species. Activation of glycogenolysis and muscle contraction by the same Ca²⁺-binding protein (troponin-C) represents a simple mechanism for synchronizing these two processes, and perhaps this is why phosphorylase kinase is so large (molecular weight 1,300,000 ref. 17)), having to span the distance between the protein–glycogen complex and thin filaments if these ideas are correct. Knowledge of its precise intracellular location in contracting muscle will be required to test this hypothesis.

Hormonal control of glycogen metabolism by multi-site phosphorylation: It was perhaps fortunate that the first example of enzyme regulation by reversible phosphorylation (activation of glycogen phosphorylase by phosphorylase kinase) should involve phosphorylation at a single site by a single protein kinase, since it greatly facilitated elucidation of the complex

but the experimental observations on which this hypothesis was based can now be explained by a quite different phenomenon³⁷.

Glycogen synthase is phosphorylated on seven serine residues by five different protein kinases, and all seven sites are phosphorylated *in vivo*. Cyclic AMP-dependent protein kinase phosphorylates sites 1a, 1b and 2 (ref. 38), phosphorylase kinase site 2 (ref. 12), glycogen synthase kinase-3 sites 3a, 3b and 3c (ref. 39), glycogen synthase kinase-4 site 2 and glycogen synthase kinase-5 site 5 (ref. 40). The organization of the sites in the polypeptide chain⁴¹ is shown in Fig. 3.

The effect of phosphorylation is to decrease the activity of glycogen synthase, although the changes in kinetic parameters are complex, and different phosphorylation sites have different effects. Phosphorylation increases the *K_m* for the substrate UDP-glucose, decreases the *K_i* for inhibitors such as inorganic phosphate and ADP, and increases the *K_a* for the activator glucose 6-phosphate⁴². Phosphorylation of sites (3a+3b+3c) influences the kinetic parameters to a greater extent than does phosphorylation of site 2 or site 1a, but the effects are additive so that even larger changes are produced when all five sites are phosphorylated^{40,43}. The phosphorylation of site 1b or site 5 does not seem to affect the kinetic properties^{38,40}.

In normally fed animals, glycogen synthase contains 3 mols of phosphate bound covalently to each subunit, with the phosphorylation of sites (3a+3b+3c) having a dominant role in determining the kinetic properties⁴⁴. Following the administration of adrenaline there are 5 mols of phosphate on each subunit of glycogen synthase^{44–46}, and the activity of the enzyme is decreased mainly by increased phosphorylation at site 2 and sites (3a+3b+3c)⁴⁴.

The finding that site 2 becomes fully phosphorylated in response to adrenaline in conditions where site 1a phosphorylation is only increased slightly was unexpected, as site 1a is phosphorylated much more rapidly than site 2 by cAMP-PrK *in vitro*, and is dephosphorylated more slowly³⁸. The increased phosphorylation of site 2 may therefore be mediated indirectly by cAMP-PrK, through the activation of phosphorylase kinase (Fig. 3). Even more surprising was the finding that almost half of the increase in phosphate occurs at sites (3a+3b+3c), which are phosphorylated by glycogen synthase kinase-3. This enzyme is unaffected by cyclic AMP⁴³ and does not seem to be a substrate for cAMP-PrK (B. A. Hemmings and P. Cohen, unpublished work).

Recent studies indicate that the activation of glycogen synthase by insulin is largely explained by decreased phosphorylation of sites (3a+3b+3c) (P. J. Parker and P. Cohen, unpublished work). These results suggest that glycogen synthase kinase-3 may be controlled by insulin, which is particularly interesting in view of the surprising discovery that this protein kinase is also an activator of protein phosphatase-1, the major glycogen synthase phosphatase in skeletal muscle (see the following section).

Multi-site phosphorylation is a simple mechanism for greatly increasing the regulatory potential of enzymes and is being found to be a common occurrence. Phosphorylation at one site may amplify or even antagonize the effects of phosphorylation at other sites, or alter the rates at which they are phosphorylated

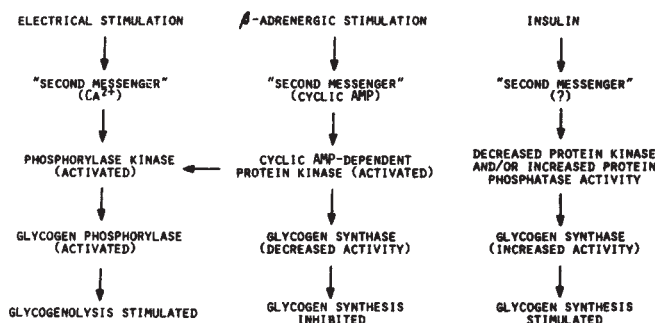


Fig. 1 Knowledge of the neural and hormonal control of glycogen metabolism in mammalian skeletal muscle up to 1970.

effects of covalent modification on the kinetic properties^{29–32}. This situation is relatively uncommon, however, and ‘multi-site phosphorylation’ is turning out to be the rule rather than the exception.

Phosphorylase kinase is phosphorylated on two serine residues by cAMP-PrK at physiological Mg²⁺ concentrations, one on the α -subunit and one on the β -subunit^{16,17}. Both serine residues become phosphorylated *in vivo* in response to adrenaline³³. Phosphorylation of the β -subunit occurs most rapidly *in vitro*, and activation correlates with the extent of phosphorylation of this subunit^{17,33,34}. Phosphorylation of the α -subunit does not alter the activity^{34,35} and its function is unclear. It was initially thought that phosphorylation of the α -subunit stimulated the rate at which the β -subunit was dephosphorylated^{34,36},

or dephosphorylated. The mitochondrial pyruvate dehydrogenase complex⁴⁷⁻⁴⁹ and retinal membrane protein rhodopsin^{50,51} are well established examples of this phenomenon. The phosphorylation of three serine residues on the α -subunit of pyruvate dehydrogenase may help to lock this enzyme into the inactive state in conditions such as insulin deprivation, thereby preventing the oxidation of glucose or its conversion to fatty acids. The phosphorylation of five serine and threonine residues at the C-terminus of rhodopsin may be involved in the regulation of dark-light adaptation. Phosphorylation at different sites by different protein kinases enables enzymes to respond to several physiological stimuli, and in such situations interactions between phosphorylation sites may frequently represent the mechanism by which one stimulus influences another. A number of examples which illustrate this idea will be described below.

Protein phosphatases involved in glycogen metabolism in skeletal muscle: Four distinct enzymes, termed protein phosphatase-1 and protein phosphatases-2A, -2B and -2C (Table 1), have been identified in mammalian skeletal muscle and other tissues that are capable of dephosphorylating the enzymes of glycogen metabolism⁵²⁻⁵⁵. Protein phosphatase-1 specifically dephosphorylates the β -subunit of phosphorylase kinase and is powerfully inhibited by two proteins termed inhibitor-1 and inhibitor-2 (see below), whereas the three type-2 protein phos-

that this activation system operates *in vivo*, an attractive idea is that it might be under the control of insulin⁵.

A further mechanism by which protein phosphatase-1 is regulated is through its interaction with the two thermostable proteins inhibitor-1 and inhibitor-2^{56,62-65}. It is of special interest that, only after phosphorylation on a threonine residue by cAMP-PrK^{56,60-62,65-68} can inhibitor-1 inhibit protein phosphatase-1 (Fig. 4). By such means inhibitor-1 provides a mechanism for amplifying the effects of cyclic AMP and for exerting control over several protein kinase-protein phosphatase cycles. Inhibitor-1 may also allow cyclic AMP to control enzymes that are phosphorylated by protein kinases other than cAMP-PrK. This would be the simplest explanation for the increased phosphorylation of glycogen synthase at sites (3a+3b+3c) in response to adrenaline, and may also contribute to some of the increased phosphorylation at site 2.

Interestingly, inhibitor-1 does not inhibit its own dephosphorylation by protein phosphatase-1⁶⁴; consequently, protein phosphatase-1 could dephosphorylate inhibitor-1 *in vivo*. However, protein phosphatases 2A and 2B are also powerful inhibitor-1 phosphatases *in vitro*. It has recently been discovered that protein phosphatase-2B is a Ca^{2+} -dependent enzyme that is stimulated 10–20-fold by calmodulin^{69,70}, suggesting that it may only be active during muscle contraction. The dephosphorylation of inhibitor-1 by protein phosphatase-2B, if it occurred *in vivo*, might therefore represent a mechanism for the activation of protein phosphatase-1 during contraction. This should stimulate the rate at which glycogen is resynthesized when contraction ceases, and allow the rate of glycogen synthesis in resting muscle to be linked to the strength and duration of the previous contraction.

Role of regulatory proteins of glycogen metabolism in other cellular processes

Cyclic AMP-dependent protein kinase: Although originally discovered as an activator of phosphorylase kinase⁴, it soon became obvious that cAMP-PrK must have a much wider role in mediating the diverse effects of cyclic AMP. The hypothesis arose⁷¹ that the specificity of a hormone is determined by whether its receptor is present on the plasma membrane of a

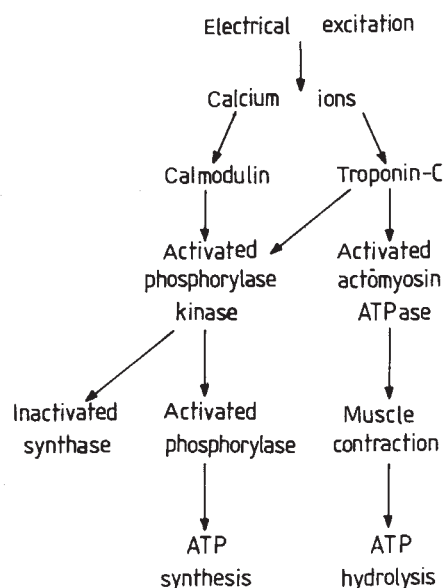


Fig. 2 Role of Ca^{2+} , calmodulin and troponin in synchronizing glycogen breakdown, glycogen synthesis and muscle contraction.

phatases preferentially dephosphorylate the α -subunit of phosphorylase kinase and are unaffected by these inhibitor proteins. The concentrations of protein phosphatases-2A and -2C are low in skeletal muscle, and account for only a small proportion of the phosphatase activity towards the enzymes of glycogen metabolism in this tissue. These two enzymes are present at higher concentrations in the liver, and are likely to be more important in the regulation of other metabolic pathways (see below).

Protein phosphatase-1 is the most important enzyme in the regulation of glycogen metabolism in skeletal muscle³⁶, where it is the major phosphatase for glycogen synthase, glycogen phosphorylase and the β -subunit of phosphorylase kinase. A single enzyme therefore carries out each of the dephosphorylation reactions that inhibit glycogenolysis or activate glycogen synthesis (Fig. 4). It is becoming increasingly clear that protein phosphatase-1 is controlled in a variety of ways. A form of the enzyme has been isolated that is completely inactive until incubated with a further protein and Mg^{2+} -ATP^{37,56-61}, and this activating protein has surprisingly turned out to be glycogen synthase kinase-3^{59,60}. Although there is not yet any evidence

Table 2 Enzymes activated by calmodulin

Enzyme	Tissue	Refs
High K_m cyclic nucleotide phosphodiesterase	Brain, heart	150–152
Adenylate cyclase	Brain	107
$(\text{Ca}^{2+} + \text{Mg}^{2+})\text{ATPase}$	Erythrocyte and other cell membranes	153, 154
Phosphorylase kinase	Muscle	19, 26, 28
Myosin kinase	Muscle and non-muscle tissues	94, 95
Glycogen synthase kinase	Liver	108
Phospholamban kinase	Cardiac muscle	109
Tryptophan/tyrosine hydroxylase kinase	Brain	110, 155–157
Protein-I kinase	Brain	111, 112
Protein phosphatase-2B	Muscle, brain	70
NAD kinase	Sea urchin eggs, higher plants	158, 159
Guanylate cyclase	<i>Tetrahymena</i>	160

target cell, and which physiological substrates for cAMP-PrK are present within those cells. In the case of substrates that are enzymes, the effect of phosphorylation is often to change the K_m for a substrate, the K_a for an activator or the K_i for an inhibitor, as clearly illustrated by the enzymes of glycogen metabolism. Substrates, activators and inhibitors may also affect the rate at which an enzyme is phosphorylated or dephosphorylated, thereby amplifying or suppressing the effects of covalent modification. Phosphorylation-dephosphorylation should not therefore be regarded as a mechanism for turning an enzyme

on or off but rather for switching it between two forms (or up to several hundred in the case of enzymes with complex subunit structures that exhibit multi-site phosphorylation) that respond differently to substrates and regulator molecules. The interplay between covalent phosphorylation, allosteric effector molecules and substrates represents the means by which extracellular (neural and hormonal) and intracellular information is integrated to determine the precise activity of a metabolic pathway *in vivo*.

One obvious prediction of this model is that many substrates for cAMP-PrK must exist in different cells to explain the great diversity of action of many hormones, and it has become increasingly clear that this is the case. A number of enzymes are regulated by cAMP-PrK *in vitro* and are likely to be physiological substrates for this enzyme. The activation of triglyceride lipase⁷² and inactivation of glycerol phosphate acyl transferase⁷³ may coordinate triglyceride breakdown and synthesis respectively in adipose tissue in response to adrenaline, in the same way that activation of phosphorylase kinase and inhibition of glycogen synthase allow coordinated control of glycogen breakdown and synthesis by adrenaline (skeletal muscle) or glucagon (liver). The activation of cholesterol esterase increases the pool of cholesterol for steroidogenesis in the adrenal cortex in response to ACTH stimulation⁷⁴. The phosphorylation of acetyl-CoA carboxylase decreases its activity and increases the K_a for the activator citrate⁷⁵. This underlies the inhibition of fatty acid synthesis in adipose tissue by adrenaline⁷⁶ and in the liver by glucagon⁷⁷. Acetyl-CoA carboxylase is phosphorylated at multiple sites and several protein kinases (including glycogen synthase kinase-5) seem to be involved^{40,75}. The inactivation of 6-phosphofructo 2-kinase^{78,79} and pyruvate kinase⁸⁰, and activation of fructose 1:6 biphosphatase^{81,82}, appear to be crucial events in the stimulation of gluconeogenesis by glucagon in hepatic tissue. 6-Phosphofructo 2-kinase catalyses the formation of fructose 2:6 biphosphate, a key activator of 6-phosphofructo 1-kinase and a powerful inhibitor of fructose 1:6 biphosphatase⁸³⁻⁸⁶.

The phosphorylation of pyruvate kinase increases the K_m for phosphoenolpyruvate, makes it more sensitive to the inhibitors ATP and alanine, and increases the K_a for the activator fructose 1:6 biphosphate⁸⁰. The phosphorylation of fructose 1:6 biphosphatase decreases the K_m for fructose 1:6 biphosphate⁸¹. The activation of hepatic phenylalanine hydroxylase⁸⁷⁻⁹⁰ is probably one of several mechanisms by which amino acid degradation is activated to provide gluconeogenic precursors and tricarboxylic acid cycle intermediates in response to glucagon. The activation of tyrosine hydroxylase in the adrenal gland or brain⁹¹⁻⁹³ increases the rate of synthesis of the hormones and neurotransmitters adrenaline, noradrenaline and dopamine in response to neural excitation. The Ca^{2+} -calmodulin-dependent

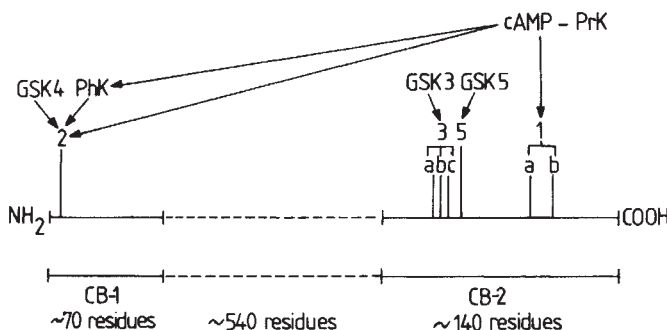


Fig. 3 Organization of the phosphorylation sites in rabbit skeletal muscle glycogen synthase. Site 2 is seven residues from the N-terminus and sites 3a, 3b, 3c, 5, 1a and 1b are 30, 34, 38, 46, 87 and 100 residues from the N-terminus of a large CNBr-peptide (CB-2) at the C-terminal end of glycogen synthase⁴¹. Abbreviations: cAMP-PrK, cyclic AMP-dependent protein kinase; PhK, phosphorylase kinase; GSK, glycogen synthase kinase.

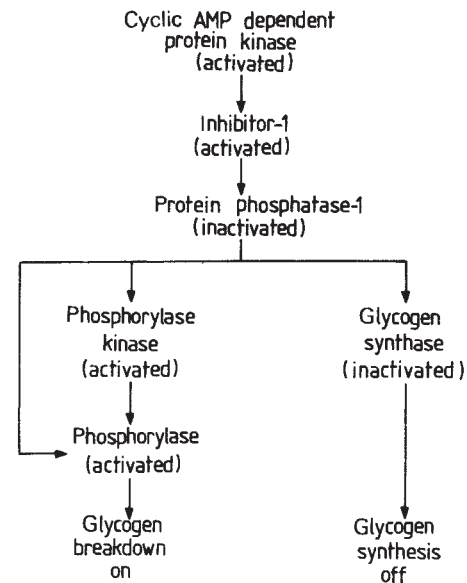


Fig. 4 Role of protein phosphatase-1 and inhibitor-1 in the β -adrenergic control of glycogen metabolism in mammalian skeletal muscle.

enzyme myosin light chain kinase is essential for the assembly of myosin into filaments and for actin-activated actomyosin ATPase activity in smooth muscle and non-muscle tissues⁹⁴⁻⁹⁶. The inactivation of this enzyme by cAMP-PrK⁹⁷ may be a key event in the adrenaline-induced relaxation of smooth muscle^{98,99}.

Of the 12 enzymes discussed above, only phosphorylase kinase, glycogen synthase and pyruvate kinase meet all four criteria necessary to establish that a protein serves as a physiological substrate for cAMP-PrK *in vivo*^{100,101}, although acetyl-CoA carboxylase⁷⁶ and phenylalanine hydroxylase^{89,90} come very close to doing so.

Although it is likely that regulation by hormones of processes such as muscle contractility, secretion, membrane permeability and transport, growth and differentiation, protein induction and protein degradation also involve phosphorylation, progress is still hampered by a lack of understanding of the molecular nature of the processes themselves. Nevertheless, the importance of cAMP-PrK has been established in several cases. For example, the phosphorylation of troponin-I in cardiac muscle fibres decreases the affinity of troponin-C for Ca^{2+} , and may contribute to the increased rate of relaxation of cardiac muscle produced by adrenaline⁹⁵. Similarly, the phosphorylation of a protein in cardiac sarcoplasmic reticulum, termed phospholamban, is associated with activation of the sarcoplasmic reticulum ATPase and increased rates of Ca^{2+} uptake into these vesicles^{95,102-104}. This may also promote the increased rate of relaxation of cardiac muscle by adrenaline. The growth of mammalian cells is inhibited by cyclic AMP after several days in culture, allowing the selection of mutant strains in which this phenomenon no longer occurs, and these mutant cells are invariably found to have defective cAMP-PrK activity^{105,106}. Thus, even the long-term effects of cyclic AMP on cell growth are clearly mediated by this protein kinase, and must involve the phosphorylation of as yet unidentified proteins.

It is important to stress that cAMP-PrK is a highly specific enzyme, phosphorylating very few proteins at significant rates. Even physiological substrates are only phosphorylated at one or two sites out of a large number of serine or threonine residues that are potentially available¹⁰¹. The basis for this specificity has become clearer following the discovery that physiological substrates invariably contain two adjacent basic amino acids just N-terminal to the residue that is phosphorylated. This structural feature is now known to be critical for specific substrate recognition (reviewed in refs 5, 36, 80, 101).

Calmodulin: Neural and hormonal stimulation of a variety of cells produces transient rises in the cytoplasmic concentration of Ca^{2+} , but only recently has it become clear that many of the biological actions of Ca^{2+} are mediated by the Ca^{2+} -binding protein calmodulin, in a manner that closely resembles the action of cyclic AMP^{23,107}. The ability of calmodulin to function as a Ca^{2+} -dependent regulator of enzyme activity is a consequence of conformational changes following its binding of Ca^{2+} , which lead to the formation of specific interaction domains. Table 2 lists 12 enzymes that are activated by, or completely dependent on, calmodulin. These include a number of protein kinases or protein phosphatases, three of which (phosphorylase kinase, myosin light chain kinase and protein phosphatase-2B) have been discussed already. A calmodulin-dependent protein kinase in rat liver phosphorylates site 2 of glycogen synthase, but is distinct from phosphorylase kinase as it cannot activate glycogen phosphorylase¹⁰⁸. A cardiac protein kinase which phosphorylates phospholamban does so at a site(s) distinct from that phosphorylated by cAMP-PrK, and is associated with increased rates of Ca^{2+} uptake¹⁰⁹. A brain protein kinase phosphorylates tryptophan hydroxylase and tyrosine hydroxylase¹¹⁰, and therefore regulates the synthesis of the neurotransmitters serotonin and dopamine. Protein-I, a major protein of the postsynaptic membrane, that may function in the control of neurotransmitter release, is a target for two different calcium-dependent protein kinases that phosphorylate distinct serine residues on the protein. Protein-I is also a target for cAMP-PrK^{111,112}. In addition, a variety of tissues contain membrane-bound calmodulin-dependent protein kinases that phosphorylate membrane proteins whose identity and function are as yet unknown^{113,114}.

Although the cyclic AMP and calmodulin systems are formally analogous in the way in which they produce phosphorylation of a number of proteins (Fig. 5), a major difference between the two systems is that mammalian cells contain one catalytic subunit of cAMP-PrK that phosphorylates a variety of proteins¹⁰¹, whereas many different protein kinases with restricted substrate specificities are activated by calmodulin.

Another point, indicated by the broken lines in Fig. 5, is that the cyclic AMP and Ca^{2+} -calmodulin pathways are closely interlinked and may often be interchangeable in different tissues. Thus, adrenergic stimulation of glycogenolysis in skeletal muscle involves the β -receptors and is mediated by cyclic

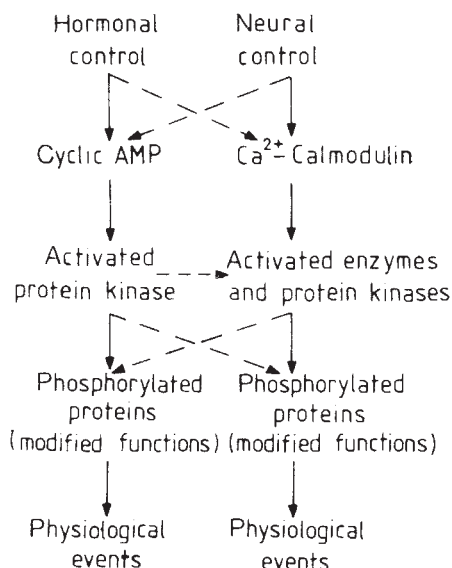


Fig. 5 Interrelationships between the cyclic AMP and Ca^{2+} -calmodulin systems in the neural and hormonal control of cellular activity.

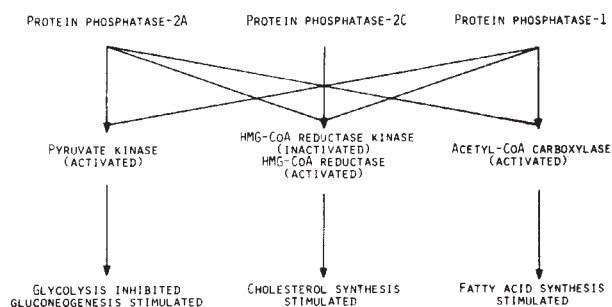


Fig. 6 Role of protein phosphatases-1, -2A and -2C in the regulation of gluconeogenesis, cholesterol synthesis and fatty acid synthesis.

AMP, whereas in adult rat liver it is an α -adrenergic effect mediated at least in part by Ca^{2+} (refs 115, 116). Similarly, electrical excitation of skeletal muscle elevates the cytoplasmic Ca^{2+} concentration, whereas it increases cyclic AMP as well as Ca^{2+} in the brain. The latter effect may result from activation of the calmodulin-dependent adenylate cyclase (Table 2) which is present in brain, but not in most other tissues.

A second point of interconnection between the cyclic AMP and Ca^{2+} -calmodulin pathways is at the level of the protein kinases. Figure 5 suggests that cAMP-PrK may often phosphorylate calmodulin-dependent enzymes. The two well established examples of this phenomenon are phosphorylase kinase and myosin light-chain kinase. Further interconnection also occurs at the level of phosphorylated substrates. Figure 5 suggests that cyclic AMP and Ca^{2+} -calmodulin-dependent protein kinases frequently phosphorylate the same proteins, although this will usually occur at distinct sites due to the high specificity of protein kinases (multi-site phosphorylation). Examples already discussed here are glycogen synthase, phospholamban, tyrosine hydroxylase and protein-I. Another example is 'goblin', a protein of molecular weight 230,000 in the plasma membrane of avian erythrocytes, that is implicated in the hormonal regulation of Na^{+} - K^{+} co-transport¹¹⁷.

Although the importance of calmodulin in cellular regulation is firmly established, many fundamental questions remain unresolved. For example, the ability of calmodulin to activate an enzyme *in vitro* does not prove that this is a physiologically relevant reaction, and calmodulin may simply be substituting for another calcium-binding protein. Another question is whether different calmodulin-dependent enzymes bind to calmodulin in an identical manner, and if they do not¹¹⁸, might one molecule of calmodulin be able to activate several different enzymes simultaneously? Are different calmodulin-dependent enzymes activated by the same concentration of Ca^{2+} *in vivo*? This will depend not only on how many Ca^{2+} -binding sites must be occupied in order to achieve activation, but on the amount of free calmodulin available to interact with any given enzyme^{25,119}, and on the changes in the Ca^{2+} -binding properties of calmodulin that accompany its interaction with target proteins. Several calmodulin-dependent enzymes are apparently only activated when all four Ca^{2+} -binding sites on calmodulin are fully occupied^{23,119}, but this requirement could be modified by phosphorylation or some other covalent modification as in the case of phosphorylase kinase. Note also that one enzyme can be regulated by two different Ca^{2+} -binding proteins; this is the case for phosphorylase kinase and protein phosphatase-2B⁷⁰ and may apply frequently. All these possibilities demonstrate that the potential for very sophisticated control by Ca^{2+} exists; this could, in a sense, be regarded as the equivalent of multi-site phosphorylation.

The protein phosphatases involved in cellular regulation: One of the first pieces of evidence that implicated cAMP-PrK in metabolic processes other than glycogen metabolism, was its

high concentration in tissues where glycogen metabolism was of very minor importance. This is also the case for protein phosphatases-1, -2A, -2B and -2C in mammalian tissues^{120,121}. Indeed, current evidence suggests that protein phosphatases-2A, -2B and -2C may be more important in the control of metabolic pathways other than glycogen metabolism.

Protein phosphatases-1, -2A and -2C (Table 1) have broad substrate specificities⁵³⁻⁵⁵, and account for all the measurable protein phosphatase activity in mammalian liver towards L-pyruvate kinase, acetyl-CoA carboxylase, hydroxymethylglutaryl-CoA (HMG-CoA) reductase and HMG-CoA reductase kinase¹²²⁻¹²⁴, as well as the enzymes of glycogen metabolism. They therefore seem to be the major protein phosphatases involved in the control of gluconeogenesis, fatty acid synthesis and cholesterol synthesis (Fig. 6). The concentrations of protein phosphatases-2A and -2C are about threefold higher in liver than skeletal muscle^{55,121}, supporting the idea that they may be particularly important in the regulation of hepatic metabolism.

Protein phosphatases-2A and -2C have been isolated from smooth muscle as myosin light-chain phosphatases^{125,126}, and may be involved in the regulation of muscle contraction. However, protein phosphatase-1 and protein phosphatase-2B are also myosin light-chain phosphatases *in vitro*^{54,69}. Protein phosphatases-1 and -2A seem to be the major phosphatases acting on protein synthesis initiation factor eIF-2^{54,126} and may therefore function as stimulators of protein synthesis¹²⁷.

In view of the broad and overlapping substrate specificities of protein phosphatases-1, -2A and -2C *in vitro*, it is frequently difficult to assess the relative importance of these enzymes in the regulation of different metabolic pathways. In skeletal muscle, the concentrations of protein phosphatase-2A and protein phosphatase-2C are low, and it is difficult to believe that protein phosphatase-1 is not the major protein phosphatase involved in glycogen metabolism, particularly as it is specifically associated with glycogen. In the liver, the situation is much less clear. Protein phosphatase-1 is not only associated with glycogen⁵⁵, but also with microsomes¹²¹, suggesting an involvement in processes such as cholesterol synthesis and protein synthesis. Protein phosphatases-2A and -2C are exclusively located in the liver cytosol^{55,121}. The former is very active towards the enzymes that regulate gluconeogenesis, fatty acid synthesis and cholesterol synthesis¹²², while the latter is most active against the enzymes of cholesterol synthesis^{54,122}. However, these observations do not prove that they are the most important protein phosphatases acting on these substrates *in vivo*.

It was recently reported that microinjection of inhibitor-1 blocks the progesterone-induced division of *Xenopus* oocytes¹²⁸, suggesting that protein phosphatase-1 is responsible for a dephosphorylation event that is known to trigger the meiotic maturation of oocytes¹²⁹. Introduction of inhibitor-1 and inhibitor-2 into other cells could prove to be a powerful method for probing the biological functions of protein phosphatase-1.

The Ca²⁺-calmodulin-dependent protein phosphatase, protein phosphatase-2B (Table 1), is present at high concentrations in skeletal muscle and brain, but its concentration is low in liver^{55,121}. It appears to be identical to calcineurin (also termed CaM-BP₈₀), a major calmodulin-binding protein in brain^{70,130,131}, found in particularly high levels in the neostriatum¹³¹. Its immunohistochemical localization at postsynaptic densities and microtubules of postsynaptic dendrites¹³¹ suggests a role in the regulation of neurotransmitter action and microtubular function.

Protein phosphorylation and the control of cellular activity

The preceding sections have summarized some of the major lines of evidence that implicate cAMP-PrK, calmodulin and protein phosphatases-1, -2A, -2B and -2C in cellular processes

other than glycogen metabolism. Inhibitor-1, inhibitor-2 and glycogen synthase kinase-3 should also have more general roles as they control the activity of protein phosphatase-1. Thus, a wide range of cellular processes may respond to neural and hormonal stimuli by using remarkably similar regulatory networks that share common protein kinases, protein phosphatases and regulatory proteins.

Of course, many questions are far from being resolved. One concerns the role of 'silent' phosphorylation sites that do not appear to influence enzyme activities directly, such as the α -subunit of phosphorylase kinase or sites 1b and 5 of glycogen synthase. Two further examples are 6-phosphofructo 1-kinase^{132,133} and ATP-citrate lyase^{134,135} in rat liver. These enzymes are substrates for cAMP-PrK *in vitro* and *in vivo*, yet their functional significance remains unclear. These phosphorylations could, nevertheless, have important roles; for example, they might regulate turnover or alter the ability of these enzymes to interact with other proteins or regulatory molecules.

The most important outstanding problems concern the mechanism of action of hormones such as insulin or epidermal growth factor (EGF) (see refs 5, 136-138 for reviews), that do not appear to use cyclic AMP or Ca²⁺ as second messengers. The interaction of insulin with its receptor in the plasma membrane is known to trigger both increased and decreased phosphorylation of various intracellular proteins, and this hormone is believed to generate a second messenger that regulates a protein kinase(s) and/or a protein phosphatase(s). However, the identity of the second messenger, or the interconverting enzyme(s) that would be its primary target, has remained elusive. It would seem necessary to postulate that an insulin-stimulated protein kinase activates a protein phosphatase, or vice versa, to explain increased and decreased phosphorylation of different proteins by a common mechanism. Alternatively, the insulin second messenger might interact with two or more interconverting enzymes¹³⁹.

A further suggestion is that the insulin-receptor interaction directly activates a protein kinase, perhaps causing its dissociation from the plasma membrane. Each molecule of this protein kinase might then be able to phosphorylate many molecules of each substrate protein, allowing amplification of the hormonal stimulus without the need for any second messenger¹³⁶. The precedent for this idea is EGF. The binding of EGF to its receptor causes the latter protein to be phosphorylated by a protein kinase that is closely associated with the receptor¹³⁸ and similar observations have recently been reported for the insulin receptor¹⁴⁰. Curiously, phosphorylation of the EGF receptor occurs on a tyrosine residue. This is most unusual, as phosphotyrosine represents only 0.03% of all phosphorylated amino acids in normal cells, phosphoserine and phosphothreonine accounting for the remaining 99.97% (refs 141, 142). The phosphotyrosine content of cells can be increased up to 10-fold following transformation by some RNA tumour viruses^{141,142} and the transforming gene of several such viruses has turned out to be a tyrosine-specific protein kinase which shows intriguing similarities to the EGF-stimulated protein kinase^{138,143-145}. These exciting developments implicate abnormal protein phosphorylation as the cause of some virally mediated cancers.

A number of physiological stimuli that do not elevate cyclic AMP, for example, muscarinic-cholinergic stimulators, vasopressin, angiotensin and α -adrenergic stimulation, induce a rapid hydrolysis of phosphatidylinositol in the plasma membranes of their target cells, to yield inositol cyclic phosphate and diacylglycerol. It is therefore of particular interest that a new type of protein kinase has been discovered which has an absolute requirement for diacylglycerol, phosphatidylserine and physiological concentrations of Ca²⁺ (refs 146, 147). This enzyme, termed protein kinase-C, exists in an inactive form in the soluble fraction of all mammalian cells, but in the presence of Ca²⁺ associates with membranes to exhibit full catalytic activity. The Ca²⁺ dependency of this enzyme seems not to be conferred by either calmodulin or related Ca²⁺-binding pro-

teins. It is attractive to speculate that protein kinase-C mediates the actions of several hormones, with diacylglycerol acting as the second messenger. Further work will be needed to test this hypothesis, although there is some evidence that protein kinase-C is involved in the thrombin-induced release of serotonin from platelets, and a protein of molecular weight 40,000 has been implicated as the major intracellular target for protein kinase-C in this cell^{146,147}.

Finally, I predict that the study of phosphorylation of proteins in the cell nucleus will become a major growth area over the next few years. Protein phosphorylation is very extensive in the

nucleus, but only in very few cases (histone H1 phosphorylation as a signal for mitosis¹⁴⁸, activation of RNA polymerase-1¹⁴⁹) have functional roles started to be defined. I have little doubt that protein phosphorylation will be found to have many crucial roles in the control of gene structure and expression, and progress will surely be facilitated by the advances being made in unravelling the structure of the mammalian chromosome.

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ARTICLES

Warm saline bottom water in the ancient ocean

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Considerable isotopic evidence indicates that oceanic bottom water was much warmer in the geological past. A simple convection model driven by multiple turbulent buoyant plumes and observations of present deep water formation have led to the development of a theory for the formation of warm saline bottom water. It is suggested that changes in the size and configuration of marginal seas in net evaporation zones due to lithospheric plate motions and eustatic sea level change caused these seas to become sources of warm saline bottom water. Evidence from the palaeotemperature record and palaeogeography during the late Cretaceous is used to reinforce the hypothesis. Climatic and chemical consequences of the formation of warm saline bottom water are discussed.

THE measurement of oxygen isotope ratios in benthic foraminifera from deep-sea cores¹ has shown that oceanic bottom water has been much warmer in the geological past than it is at present—as warm as 15 °C in the late Cretaceous (about 70 Myr BP). A simple convection model driven by turbulent buoyant plumes developed by Peterson^{2,3} has led us to develop a model for the production of warm saline bottom water (WSBW). We suggest that in the geological past, changes in the size and configuration of marginal seas in net evaporation zones, due to lithospheric plate motions and eustatic (global) sea level changes, caused these seas to become sources of WSBW.

Turbulent plume model

It has been pointed out that bottom water is formed in small regions. Rossby⁴ suggested that this is because convective buoyancy transfer processes are much more efficient than conductive processes. Thus, to achieve a steady state and hence no net buoyancy flux across the surface, the system evolves so that the convective processes occur on only a small portion of the surface. Moreover, virtually all deep water seems to be formed in semi-enclosed regions (over continental shelves and low- and high-latitude marginal seas)⁵. We suggest that water trapped in marginal seas is made sufficiently dense, by interaction with the atmosphere, for the outflow to drive a turbulent plume.⁶ A characteristic of turbulent plumes is the entrainment of environmental fluid into the plume, an idea which is supported by the observation that, although the Mediterranean water at its source is denser than any water in the Atlantic Ocean, it only penetrates to about 1,200 m. Furthermore, Smith⁷ esti-

mated that the volume flux of the Mediterranean outflow increases by as much as 10-fold before it terminates at depth and spreads throughout the North Atlantic.

Thermohaline circulation models have tended to treat deep-water formation as a tacit assumption or as a response to global scale forcing. The importance of the outflows of marginal seas as sources of deep water suggested a simple steady-state convection model driven by imposed buoyancy sources. The model is basically the filling of a large tank whose width is very much greater than the plume width scale. The plumes interact with a laterally well mixed interior by the entrainment of interior fluid into the plumes. The model is an extension of one developed by Baines and Turner⁸.

The turbulent plume model is detailed elsewhere^{2,3} but briefly, the model calculations indicate that the depth of termination and horizontal spreading of the plumes is directly related to their buoyancy flux which is the product of the density difference between the plume fluid and the surrounding environment and the volume flux of the plume. The plume with the greatest buoyancy flux will form the bottom water and plumes of lesser buoyancy flux will terminate and spread out at intermediate depths. With two plumes, the termination depth of the weaker plume is relatively insensitive to the difference in buoyancy flux between the two until they become nearly equal in strength. Time-dependent calculations have not yet been made, but it seems that if the plumes switch (that is, the bottom water plume loses buoyancy flux or the intermediate water plume gains buoyancy flux) a relatively rapid and perhaps catastrophic change occurs when the old minor plume becomes the source for the new bottom water.

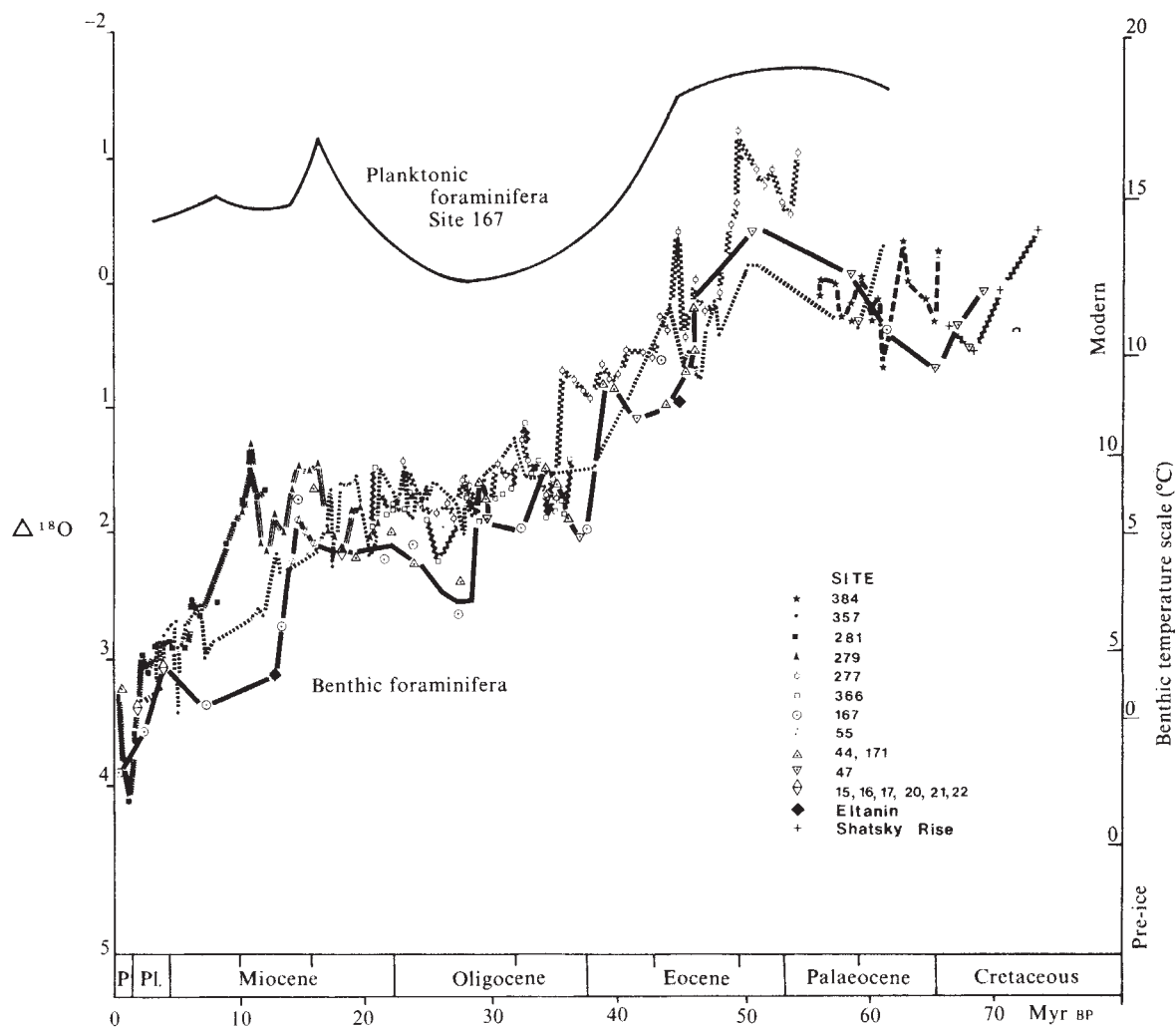


Fig. 1 Oxygen isotope palaeotemperatures from ref. 1.

Discussion

Oxygen isotopic palaeotemperature studies (on benthic foraminifera from deep-sea cores) have shown that the temperature of bottom water has decreased from $\sim 15^\circ\text{C}$ during the Cretaceous to 3°C at present (see Fig. 1). Superimposed on the generally decreasing isotopic temperature trend is a series of step changes—some of which are also found in the isotope record of pelagic foraminifera and have been interpreted as shifts in the isotopic composition of the oceans due to sudden increases in ice volumes⁸. After correction for this ice effect, residual oxygen isotope shifts remain in the deep ocean which are not reflected in the surface ocean. These discontinuities in the isotopic record may be due to hiatuses in sedimentation, or they may reflect a series of shorter time scale transitions in bottom water temperature. If these step-wise changes are confirmed, Peterson's competing plume model suggests that deep water is produced by multiple sources, both warm and cold. The temperature and other characteristics of the bottom water are determined by the deep water source area that produces the largest buoyancy flux. These source strengths have varied through time due to changes in areas, location and configuration of marginal seas due to plate motions and eustatic sea level changes. We believe that in the Cretaceous, warm water sources were dominant whereas today cold bottom water is produced at high latitudes. This change necessitated at least one transition from WSBW to cold polar bottom water. However, because of the sensitivity of plume termination depths to small differences in source strengths, several transitions might be expected as a result of variations

in buoyancy fluxes from competing deep-water sources during a period in which the source strengths were nearly the same.

A simple steady-state balance calculation shows that the buoyancy flux from a net-evaporation basin essentially depends on the evaporative flux only and hence the area of the basin and the evaporation rate, and is independent of the salinity. There may be dynamic constraints on the outflow that depend on details such as the configuration of the straits that connect the basin to the ocean; but these constraints are neglected here. We suggest that at times in the geological past, the largest buoyancy flux has emanated from marginal seas in net evaporation zones.

Palaeogeographical considerations

Eustatic sea-level fluctuations can be caused by variations in the global seafloor spreading rate⁹. The area of continent flooded by given increases in sea level is a function of the global hypsography at that time. The size, and hence drainage efficiency, of the continents directly controls the shape of the hypsographic curve¹⁰. Thus, times of increased seafloor spreading and continental breakup generate large areas of flooded continent, termed epicontinental seas, both by raising sea level and by lowering the elevation of the continents.

Epicontinental seas and marginal seas producing WSBW must be located in the zone of net evaporation ($10\text{--}40^\circ\text{N}$ and S) associated with the descending branches of the atmospheric Hadley Cell circulation. The distribution of Mesozoic and Cenozoic evaporite deposits strongly suggests that this zone has remained stationary during the past 120 Myr. Figure 2

shows the areas of flooded continents and marginal seas in 10° lat. intervals over the past 100 Myr as measured from the palaeogeographical maps of Barron *et al.*¹¹. The most dramatic change during this time has been the decrease in area of shallow and marginal seas in the high evaporation belt (10 – 40°).

Because area is an important factor determining the buoyancy flux from net-evaporation basins, the decrease in the area of net-evaporation marginal seas over the past 100 Myr strongly suggests that the late Cretaceous was much more favourable for the production of WSBW. Plots of marginal sea area against time exhibit the same trend as the oxygen isotope temperature record curve from benthic foraminifera. The decrease in area of evaporating marginal seas and the decrease in the temperature of bottom water during the Cenozoic suggests a transition in the mode of deep-water formation in which cooling at high latitudes has played an increasingly important part.

Changes in the latitudinal distribution of epicontinental and marginal seas due to the motions of the lithospheric plates also occur. This movement may transport flooded regions into or out of the net evaporation belt. These movements seem to have had little effect on the areas producing WSBW in the Mesozoic (230–65 Myr BP) and Cenozoic (65–0 Myr BP), but may have been more important in earlier times.

The occurrence of WSBW would have substantial consequences for the ocean and atmosphere. The climatic consequences^{12,13} of WSBW indicate that the suggested formation of WSBW is consistent with the equable climate of the late Cretaceous. The occurrence of a more equable climate (lesser meridional temperature gradients, with milder conditions at high latitudes) is supported by the occurrence of tropical and temperate faunas and floras, in the terrestrial record, at higher latitudes than at present.

WSBW formation complicates the interpretation of the oxygen isotope record. In the present ocean, dilution with run-off water leads to a decrease in ^{18}O . The isotopic composition of freshwater entering the northern North Atlantic is approximately -20% and in the Arctic Ocean is about -40% (ref. 14). An understanding of the temperature, salinity and oxygen isotope effects which might be caused by horizontal salinity

gradients can be derived from the consideration of a two-dimensional model ocean with a homogeneous deep water containing 90% of the total volume and a surface layer containing 10% of the total. The surface layer is, on average, 5% fresher than the deep water and ranges uniformly from undiluted at the Equator to a 10% diluted at the poles. The diluting freshwater is taken to be -20% the mean salinity and $\delta^{18}\text{O}$ for the ocean are 35‰ and 0‰ respectively. We can calculate the following: the mean $\delta^{18}\text{O}$ of the surface water is -0.9% ranging from $+0.1\%$ at the Equator to -1.9% at the poles. The error in isotopic temperature calculated on the assumption that the ocean is uniformly 0‰ ranges from -0.4°C at the Equator to $+8.0^\circ\text{C}$ at the poles. The salinity of the surface water ranges from 35.18‰ to 31.66‰ and the deep water salinity is 35.18‰. The $\delta^{18}\text{O}$ in deep water is $+0.1\%$ and the temperature error is -0.4°C . This model assumes, arbitrarily, that the surface water at the Equator has the deep water properties. A 10% reduction in the salinity of polar surface water is sufficient to prevent sinking of polar surface water into deep water at normal salinity and a temperature of 15°C regardless of the surface water temperature. In practice, the distribution of oxygen isotopes and freshwater in a real ocean are considerably more complicated than this model. In addition to such barriers to deep water flow as oceanic ridges and gateways, climatic and geographical mechanisms may vary the transport of water away from evaporating seas.

Conclusions

Our hypothesis has numerous consequences for the chemistry of the ocean and atmosphere. The chemical state of the ocean when WSBW was dominant would have been quite different from that which exists at present, and the effects should be recorded in the chemistry and isotopic composition of marine sediments. One immediate consequence follows from the fact that the solubilities of many gases strongly depend on temperature and salinity. The increased temperature of WSBW at its source (15°C compared with -2°C for present-day cold bottom water) where it is in contact with the atmosphere would reduce concentration of dissolved gases in the source water. Of particular interest are past variations in the concentrations of molecular oxygen and carbon dioxide in deep water as a consequence of WSBW. These variations should leave a signature in the accumulation rates of organic carbon and carbonate in the deep sea.

The distribution of both dissolved oxygen and carbon dioxide within the ocean is the result of exchange with the atmosphere, the formation and destruction of organic matter and physical transport processes. Respiration by animals and bacteria causes a depletion in oxygen in most subsurface ocean waters. The longer a water mass is isolated from the atmosphere the lower its oxygen content becomes; this has enabled oceanographers to use oxygen as a circulation tracer.

Anoxic conditions occur in deep water when the consumption of oxygen by organisms exceeds the rate of oxygen supply. While anoxic conditions prevail organic carbon is accumulated in the underlying sediments at a higher rate. Deep sea anoxic events have been observed in the geological record, as laminated black shales. Some authors^{16,17} have explained such events by invoking stagnation resulting from a layer of fresh or brackish surface water or by expansion of the oxygen-minimum layer by inhibited oceanic circulation. These events may also be explained as resulting from a decrease in the ventilation of the deep ocean because of reduced oxygen solubility in the source regions without requiring any change in the circulation rate.

The thermohaline circulation in the ocean is driven by the interaction of the surface ocean with the atmosphere. At low temperatures, the removal of heat is accompanied by an increase in density and the production of deep water. This is the most important source of deep water in the ocean at present. At high temperatures, the addition of heat causes evaporation

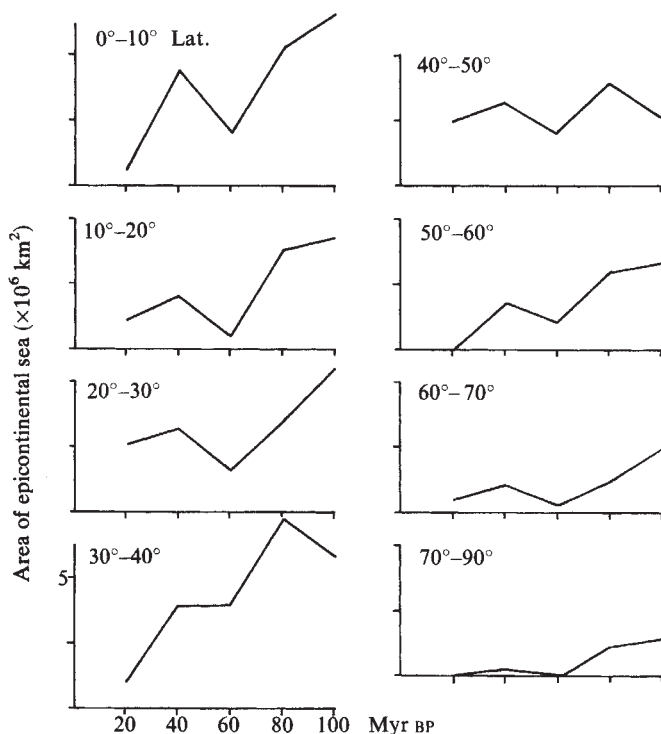


Fig. 2 Area of epicontinental seas plotted against latitude band (from the maps in ref. 11).

and an increase in salinity and density with the same results. We believe this to have been the dominant mechanism for deep water formation in historical times especially in the Cretaceous. The physics and chemistry of the process require further study as do the implications for climate and geology and especially

the suggestion that tectonic controls on sea level at various latitudes govern the mechanism of deep water formation.

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Enzymatic replication of *E. coli* chromosomal origin is bidirectional

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A soluble enzyme system has been discovered which specifically recognizes and replicates plasmids containing the Escherichia coli chromosomal origin, oriC. Electron microscopy has shown that plasmid replication begins at or near oriC from which it progresses bidirectionally to completion. Control of initiation of a cycle of chromosomal replication and mechanisms of priming and fork movement can now be explored using this system.

REPLICATION of the *Escherichia coli* K-12 chromosome, as shown by genetic and biochemical analyses *in vivo*, begins at a unique site (*oriC*) and proceeds bidirectionally¹⁻³. The DNA fragment containing *oriC* has been isolated by its ability to confer autonomous replication on plasmids or phage whose own replication origin has been inactivated⁴⁻⁷. Deletion analysis has localized *oriC* to a sequence of 232-245 base pairs (bp)⁸; insertions, deletions or substitutions in this essential region can inactivate *oriC*.

Initiation of a cycle of chromosomal replication in the cell requires the activities encoded by the genes *dnaA*, *dnaI* and *dnaP*⁹⁻¹¹. In addition, *dnaB* and *dnaC* proteins, whose activities are essential for priming the synthesis of nascent chains during replication^{12,13}, are also required during or shortly after initiation^{14,15}. RNA polymerase has also been implicated¹⁶.

A soluble enzyme system has been discovered which specifically recognizes and replicates *oriC* plasmids¹⁷. The reaction requires *dnaA* protein, RNA polymerase and numerous replication proteins including *dnaB*, single-stranded DNA-binding protein and DNA gyrase. Recently, *dnaC* protein has been shown to be required for *in vitro* replication of *oriC* plasmids (unpublished observations). Biochemical analysis of enzymatically synthesized replicative intermediates showed initiation occurring at or near *oriC* and was consistent with bidirectional progress from that point, but such evidence is essentially a statistical average. Is replication of an individual molecule, once initiated at *oriC*, then extended bidirectionally to completion? We present here the results of an electron microscopic study which indicate that with few exceptions this enzyme system creates an 'eye' or replication 'bubble' in a plasmid molecule at or near the *oriC* region which is extended in both directions to generate two complete molecules.

Formation of replicative intermediates

Two classes of supercoiled, *oriC* template DNA were examined. One, pSY317, is a 13.5-kilobase (kb) plasmid which contains a 5.6-kb *EcoRI*, *oriC* fragment (from pSY221)⁵ and a 7.9-kb

EcoRI kanamycin-resistance fragment (from pML21)¹⁸. The *oriC* plasmid contains *oriC* intact, together with extensive flanking sequences. The second template, M13*oriC*26, is a 12.2-kb, chimaeric M13 phage DNA containing *oriC* and its adjacent

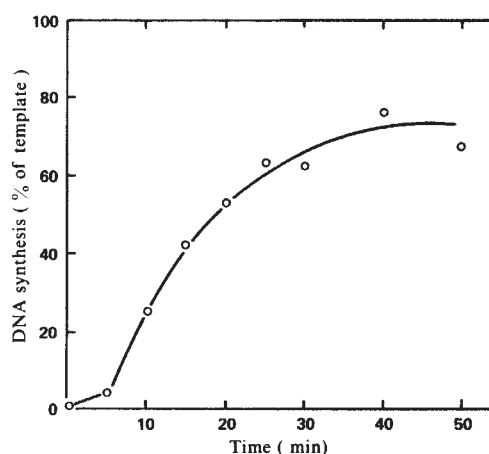


Fig. 1 Time course of *oriC* plasmid in *dnaA*-complementing conditions. Reactions were performed as described previously¹⁷ in a 250 μ l volume containing 40 mM HEPES pH 7.6, 2 mM ATP, 0.5 mM each of GTP, UTP and CTP, 43 mM creatine phosphate, 100 μ M each of dGTP, dATP, dCTP and 5-methyl-[³H]dTPP (85 c.p.m. per pmol of total deoxynucleotide), 6% (w/v) polyvinyl alcohol 24,000, 11 mM magnesium acetate, 100 μ g ml⁻¹ creatine kinase (Sigma), 8.6 μ g ml⁻¹ supercoiled pSY317 DNA, 2 mg of protein (fraction II, prepared from *E. coli* WM433 *dnaA*204 as described elsewhere¹⁷) and 230 units of *dnaA*-complementing activity (fraction III). Incubation was at 30 °C. Aliquots (25 μ l) were TCA-precipitated and counted in a liquid scintillation counter. One unit is equal to one pmol of nucleotide incorporated per min.

asnA gene¹⁹. In constructing M13oriC26, the *XhoI* site immediately to the right of *oriC* was interrupted. Although interruptions in and around this site do not alter the ability of this and similar *oriC* plasmids to replicate^{8,19,20}, the directionality of replication was reported to be affected, becoming unidirectional rather than bidirectional^{21,22}.

The extent of replication was limited to obtain a significant number of replicative intermediates for electron microscopic study. The chain terminator, 2',3'-dideoxythymidine 5'-triphosphate (ddTTP) was used. This inhibitor, which prevents chain growth when incorporated into DNA, was present at concentrations relative to dTTP that generated replicative intermediates showing different extents of replication²³. It is assumed that incorporation of a ddTMP residue in the leading or lagging strand at a replication fork stops fork movement. DNAs were purified, spread on parlodion-coated grids by the formamide

spreading technique²⁴, and rotary shadowed at a low angle with platinum-tungsten vapour. Replicative intermediates generated with an enzyme fraction (fraction II)¹⁷ from wild-type cells constituted 10–20% of the molecular forms observed, a value consistent with the fraction of template molecules used in

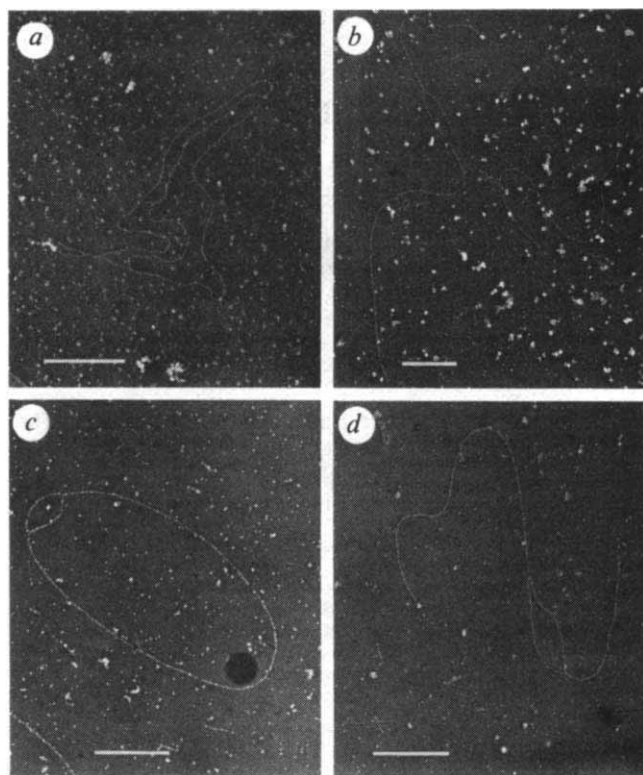


Fig. 2 Replicative intermediates of pSY317 and M13oriC26. The reaction with $8.6 \mu\text{g ml}^{-1}$ of supercoiled pSY317 was as described in Fig. 1 legend in a $125 \mu\text{l}$ volume with $50 \mu\text{M}$ ddTTP, $1,100 \mu\text{g}$ of *E. coli* WM433 *dnaA*204 fraction II and $275 \mu\text{g}$ of *E. coli* HB101 (pBF101) fraction II, known to be enriched for *dnaA*-complementation activity¹⁷. Incubation was at 30°C for 10 min after which reactions were terminated by addition of SDS to 1% and EDTA to 50 mM. Following incubation at 65°C for 3 min, aliquots were removed for TCA precipitation and counted in a liquid scintillation counter. The sample was then incubated with 0.5 mg ml^{-1} of proteinase K (Merck) at 37°C for 60 min, phenol-extracted, ether-extracted, and centrifuged in a Beckman airfuge into a CsCl shelf of 1.42 g cm^{-3} . The shelf was recovered and DNA was ethanol-precipitated and resuspended in a small volume of 10 mM Tris-HCl pH 8.0 and 1 mM EDTA. Recovery at this step was 60–70% of the initial reaction product. The sample was then incubated with $5 \mu\text{g ml}^{-1}$ of pancreatic RNase at 37°C for 60 min to digest contaminating RNA and either relaxed with *SalI* endonuclease (given by K. Burtis) in the presence of ethidium bromide³¹ (a), or restricted to completion with *SalI* endonuclease in 10 mM Tris-HCl pH 8.0, 10 mM MgSO_4 and 100 mM NaCl at 37°C for 30 min (b). DNA was then spread on to parlodion-coated grids by the formamide technique²⁴, shadowed at a low angle with platinum-tungsten vapour, and viewed in a Philips 300 electron microscope. Replicative intermediates of M13oriC26 were prepared for electron microscopy as described above except that the sample was either relaxed with *EcoRI* endonuclease (given by C. Mann) (c) or linearized by digestion to completion with *EcoRI* endonuclease in 100 mM Tris-HCl pH 7.2, 5 mM MgCl_2 , 2 mM 2-mercaptoethanol and 50 mM NaCl at 37°C for 60 min (d). Scale bars, $0.5 \mu\text{m}$.

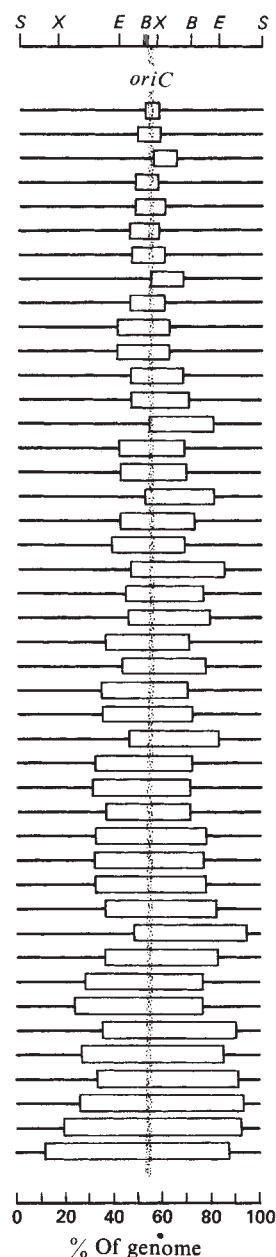


Fig. 3 Replication of the *oriC* plasmid pSY317 initiates at *oriC* and progresses bidirectionally. Replicative intermediates generated with $50 \mu\text{M}$ ddTTP, then purified and linearized with *SalI* endonuclease (as described in Fig. 2 legend), were randomly selected and photographed. Molecules were analysed with a Hewlett-Packard 9810A calculator by measuring the non-replicated and both replicated segments of an individual molecule on a Hewlett-Packard 9864A digitizer board, averaging the replicated segments, and expressing each portion as a percentage of the total length. Only molecules in which both replicated segments were identical in length ($\pm 5\%$) and for which the total length was within $\pm 10\%$ of the length expected were included in the analysis. Molecules are aligned so that the longer unreplicated segment is to the left, and they are arranged according to increasing extents of replication. The position of *oriC* is indicated by the stippled area. The open boxes represent the replication 'bubble'. Restriction endonuclease sites are: S, *SalI*; X, *XhoI*; E, *EcoRI*; and B, *BamHI*.

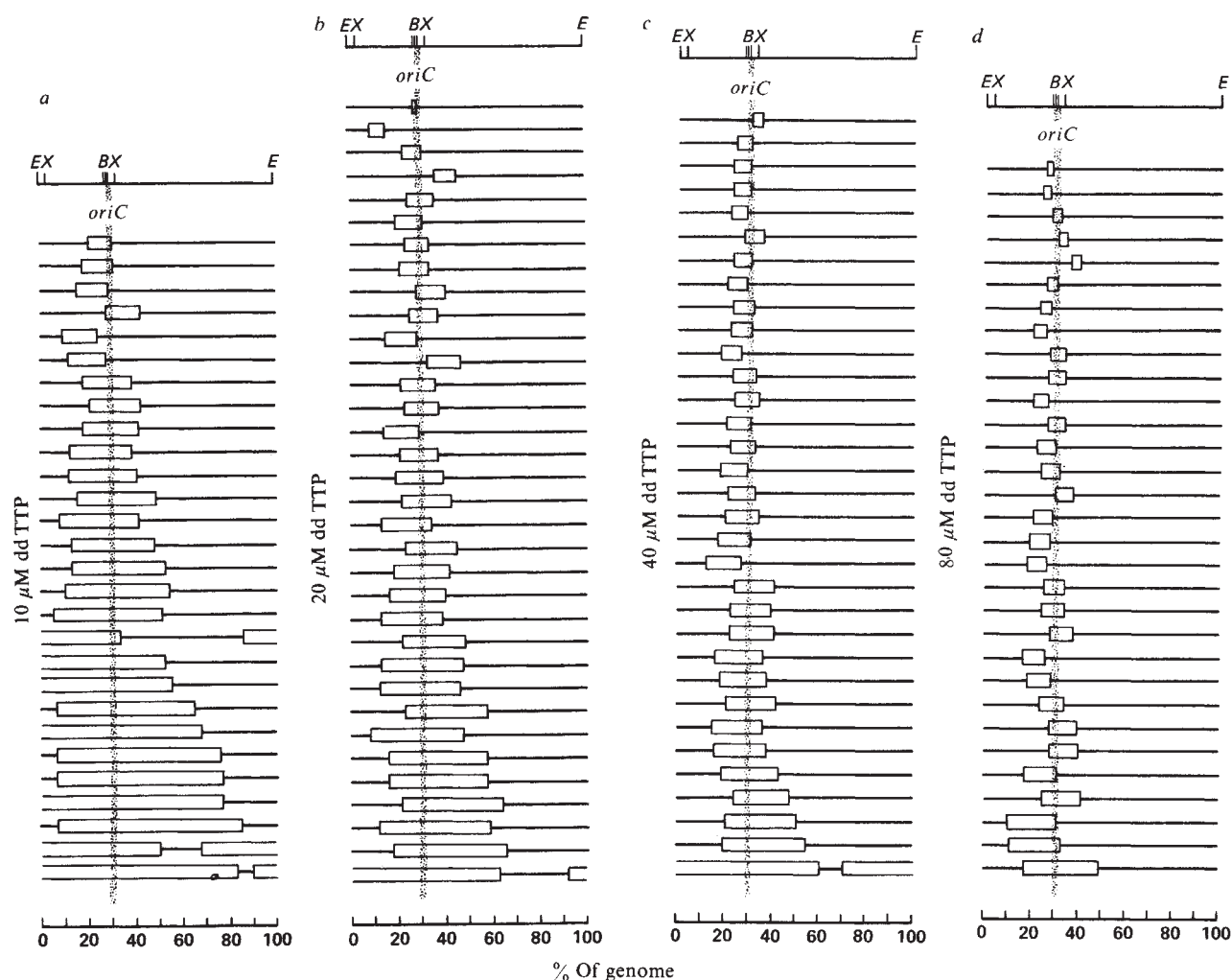


Fig. 4 Replication of M13oriC26 DNA initiates at *oriC* and progresses bidirectionally. Reactions were performed as described in Fig. 1 legend in a volume of 125 μ l containing ddTTP (as indicated) and 1 mg of enzyme fraction II prepared from *E. coli* C600 as described elsewhere¹⁷. Incubation was at 30°C for 15 min. Replicative intermediates produced with a, 10 μ M; b, 20 μ M; c, 40 μ M, and d, 80 μ M ddTTP from samples prepared as described in Fig. 2 were restricted, after treatment with pancreatic RNase, with an excess of *Eco*RI endonuclease, spread on to parlodion-coated grids and examined. Replicative intermediates at each ddTTP concentration were selected randomly, photographed and measured. Only molecules having a correct total length ($\pm 10\%$) in which both replicated segments were the same length ($\pm 5\%$) were included in the analysis. Molecules are aligned so that the longer unreplicated segment is to the right, and they are arranged according to extent of replication. The position of *oriC* is indicated by the stippled area. The open boxes represent the replication 'bubble'. Restriction endonuclease sites are the same as for Fig. 3.

comparable uninhibited reactions. It is possible that the fortuitous arrangement of a DNA fragment overlapping a circular molecule or unit-length linear fragment can give rise to structures that are indistinguishable from true replicative intermediates, but such events are expected to occur only rarely. Circular replicative intermediates of pSY317 and M13oriC26 appeared in every case as theta-like structures (see Fig. 2).

Defining the initiation site and direction of replication

Replication of an *oriC* plasmid (for example pSY317) requires *dnaA* protein¹⁷. Complementation of a crude enzyme fraction (fraction II)¹⁷ prepared from a *dnaA* mutant provides an assay for purifying *dnaA* protein overproduced in cells bearing a plasmid carrying the *dnaA* gene. In a *dnaA* protein preparation enriched at least 200-fold over wild-type levels, the template molecules were almost completely used in a reaction (Fig. 1).

In conditions which complement *dnaA* activity, replicative intermediates of pSY317 were accumulated by inhibiting the reaction by 60% with 50 μ M ddTTP. To determine the site of initiation and direction of replication, molecules were linearized

by cleavage with *Sal*I endonuclease and examined by electron microscopy (Figs 2, 3). In orienting the molecules in Fig. 3, *oriC* is located at a point 45% of the genome length from the right end of the linearized pSY317. With few exceptions, the replicated segment of the intermediate overlaps *oriC*. Although the slight asymmetry of the *Sal*I restriction cleavage relative to *oriC* introduces some ambiguity in orienting the more extensively replicated intermediates, it is clear that for most of them, replication proceeds bidirectionally. Inspection of the less extensively replicated molecules reveals that initiation occurs at or near *oriC*. Thus, we have shown that in these conditions, replication of the *oriC* plasmid pSY317 proceeds bidirectionally from a start in the *oriC* region.

Replicative intermediates of M13oriC26 DNA were generated with an enzyme fraction (fraction II)¹⁷ from wild-type cells at several levels of ddTTP inhibition and analysed after cleavage with *Eco*RI endonuclease (Figs 2, 4). In uninhibited reactions, no replication occurred from the M13 origin¹⁷ due to the absence of the M13-encoded gene 2 protein required for replication of supercoiled M13 DNA²⁵⁻²⁷. Inhibition of replication as measured by nucleotide incorporation correlated well with the effect of ddTTP in decreasing the average length of the

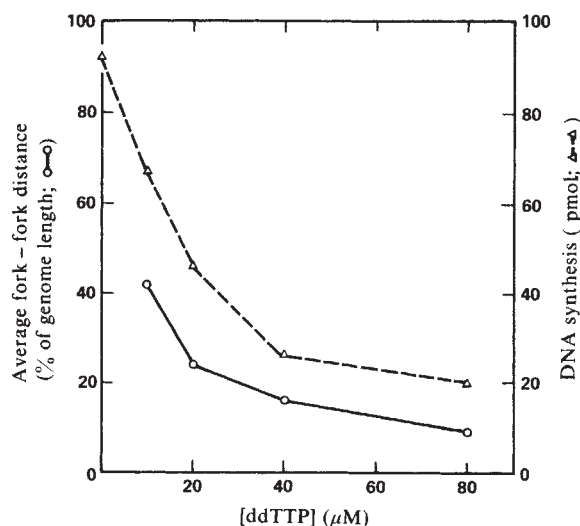


Fig. 5 Inhibition of DNA synthesis by the chain terminator ddTTP correlates with the fork-to-fork distance of replicative intermediates of M13oriC26. After incubation, aliquots (25 μl) from the reactions described in Fig. 4 legend were removed for TCA precipitation and counted to determine the amount of DNA synthesis. The replicative intermediates of Fig. 4 were then analysed individually for the extent of replication and averaged. Each point represents the average fork-to-fork distance of 28–34 replicative intermediates generated at the corresponding ddTTP concentration.

replicated segment (Fig. 5). These findings and similar results for pSY317 (data not shown) confirm that the 'eye' and 'Y' forms (molecules with a single fork) are replication intermediates.

The minimal *oriC* sequence is ~30% of the genome length from one end of M13oriC26 DNA linearized by *EcoRI* endonuclease (Fig. 4). Recombinant M13 phage DNAs from which *oriC* has been deleted do not replicate from *oriC* either *in vitro*

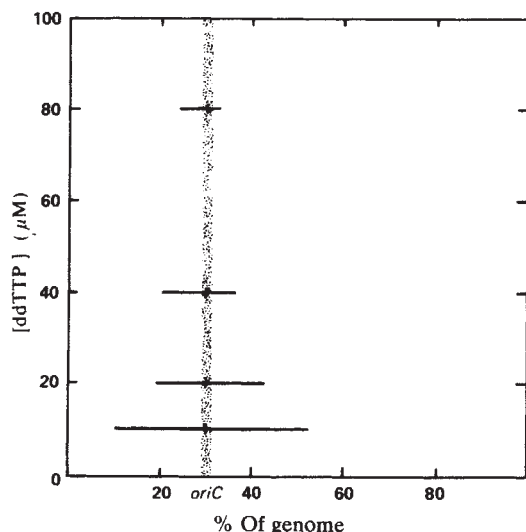


Fig. 6 Replication of M13oriC26 DNA is bidirectional for a population of molecules. Replicative intermediates at each ddTTP concentration shown in Fig. 4 were analysed individually for the extent of replication rightwards and leftwards from *oriC*. The values were averaged and plotted relative to the position of *oriC*, indicated by the stippled area, and to the physical map of M13oriC26 linearized at the single *EcoRI* site. Molecules (4 of 126) which did not seem to have initiated from *oriC* were excluded from the analysis.

or *in vivo*^{17,28}. Molecules aligned in Fig. 4 with the longer replicated segment to the right are consistent with replication initiating from the *oriC* segment. More extensively replicated molecules clearly indicate that replication proceeds bidirectionally in most cases, and the less extensively replicated molecules show initiation to be at or near *oriC*.

Replicative intermediates of M13oriC26 (Fig. 4) were analysed individually with respect to the extent of replication to the right and left of *oriC*. These values were averaged for each ddTTP concentration and plotted as the distance rightwards or leftwards relative to *oriC* (Fig. 6). The results indicate that for a population of molecules, replication from *oriC* is bidirectional.

Of 126 M13oriC26 replicative intermediates examined, 4 were judged not to have been initiated at *oriC* as the replicated segment did not overlap *oriC* within the error of measurement (5% of the genome length; Fig. 4). These discrepancies may be due to aberrant initiation or improper breakage of the duplex template. For several molecules of both template DNAs, the bidirectional progress of replication appears asymmetric or even unidirectional, which may be due to asynchrony in the initiation of the two replication forks or in chain terminations by ddTTP. In this regard, examination of the replicative intermediates of phage λ and F plasmids, which are known to replicate bidirectionally, also reveals molecules that replicate unidirectionally^{29,30}.

The bidirectional replication of M13oriC26, a template interrupted at the *XhoI* site, contrasts with the analysis of replicative intermediates of the *oriC* plasmids²⁰, pOC24, produced *in vivo*, in which predominantly unidirectional replication was attributed to an interruption at the same *XhoI* site^{21,22}. This discrepancy may be due to differences in either the plasmid sequences or the experimental conditions; in the *in vivo* study, replicative intermediates were detected at a frequency of only 1 in 10⁴ plasmid molecules and thus may not represent the principle mode of replication²¹.

This electron microscopic study of individual plasmid molecules replicated in a soluble enzyme system is thus consistent with initiation of replication from *oriC*. Fork movement from that point progresses bidirectionally. The soluble enzymatic system used here and elsewhere¹⁷ should allow a biochemical approach to the events of priming, fork movement, regulation of initiation and other events occurring at the *E. coli* origin.

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Linkage of β -thalassaemia mutations and β -globin gene polymorphisms with DNA polymorphisms in human β -globin gene cluster

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Combined analysis of DNA polymorphisms in the human β -globin gene cluster and in cloned β -genes has revealed the association of specific β -thalassaemia mutations and β -gene polymorphisms with particular flanking polymorphisms. A systematic study of cloned genes identified several new mutations, one of which possibly affects transcription. The strategy used may be applicable to other diseases of single-copy genes.

METHODS of recombinant DNA analysis, including restriction endonuclease mapping, gene cloning, DNA sequencing and gene expression assays, now permit detailed study of the molecular basis of human genetic disease. Among the first disorders examined in this context were the β -thalassaemias, inherited conditions affecting synthesis of the β -globin chain of adult haemoglobin^{1,2}. Recently, several types of specific β -globin gene defects have been defined in affected individuals, including deletion of the terminal end of the gene in Asian Indians^{3–5}, chain termination (or nonsense) mutations in Chinese and Mediterraneans^{6–9}, a frameshift deletion in a Turkish patient⁷, a point mutation within an intervening sequence (IVS) in Mediterraneans^{10,11}, and an IVS splice junction mutation in some Middle East individuals¹² and a Mediterranean (R. H. Treisman, N. Proudfoot, M. Shander and T. Maniatis, in preparation). The disparate β -thalassaemia genes analysed so far have been largely unselected or at best chosen for study on the basis of their phenotype, either β^0 or β^+ , corresponding to absent or reduced β -globin production. Using this rather random approach, the full spectrum of DNA lesions responsible for a genetic disorder may not be apparent, as cloning of mutant genes from individuals having similar phenotypes is naturally biased towards the study of the most prevalent alleles. Without using a directed approach for selection of genes for further examination, the search for new, and potentially informative, mutations is encumbered by repeated analysis of previously characterized genes.

In this study, we have developed and applied a new strategy for the comprehensive analysis of existing mutations in a class of human disease. Specifically, we have combined analysis of various restriction enzyme polymorphisms in the β -globin gene cluster with direct examination of β -globin structural genes in Mediterranean individuals having β -thalassaemia. The basis of

this approach is our finding that specific mutant genes are strongly linked to patterns of restriction site polymorphism (haplotypes) in this region of the genome. Systematic characterization of genes representing all haplotypes in an extensive panel has revealed several new mutations and the nature of sequence polymorphisms within the β -globin gene itself. This approach to the study of β -thalassaemia should be readily applicable to the systematic analysis of specific mutations of other single-copy genes which result in human disease.

Restriction site polymorphisms in β -thalassaemia DNA

Several restriction site polymorphisms distributed over more than 60 kilobases (kb) of DNA within the human β -globin gene cluster have been described^{13–17}. In an attempt to characterize in detail the DNA regions in which mutant β -globin genes lie, we have assessed cleavage at seven of these polymorphic sites in a panel of 91 β -thalassaemia chromosomes of Mediterranean origin. Nine different cleavage patterns, one of which was found along each chromosome, were observed and designated haplotypes I–IX (Fig. 1). Individual haplotypes accounted for 1–47% of the thalassaemia chromosomes studied. When analysing the same sites in normal DNA samples, we observed non-random association of the five sites located 5' to the β -globin gene (*HincII* and *HindIII* sites) and non-random association of the two sites located 3' to the δ -globin gene (the *AvaII* and *BamHI* sites)¹⁶. However, the 5' and 3' collections of sites appeared to associate independently to generate the various haplotypes¹⁶. In general, the polymorphic patterns in the thalassaemic DNA samples did not differ in frequency from patterns seen in normal individuals.

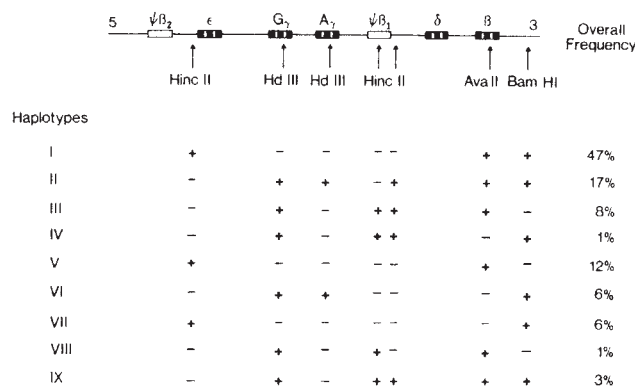


Fig. 1 Chromosome haplotypes in β -thalassaemia. Polymorphic restriction enzyme sites in the β -globin gene cluster that were used here are shown at the top in a schematic representation of the β -gene cluster. A total of 91 chromosomes of Mediterraneans with β -thalassaemia were examined at these sites by Southern³⁴ gel blotting, using methods and probes described elsewhere¹⁶. Patients were of both β^0 and β^+ phenotypes. + Indicates the presence of cleavage at a particular site, and - indicates absence of cleavage. Haplotypes indicate the patterns of cleavage along a chromosome and are arbitrarily numbered I-IX. Hd III, *Hind*III. ϵ , γ , δ and β are the expressed genes of the cluster³⁵. $\psi\beta_2$ and $\psi\beta_1$ are pseudogenes identified by gene cloning³⁵.

On the one hand, haplotypes might reflect the presence of specific β -thalassaemia alleles in a population, in which case they would mirror the chromosomal background on which a mutation arose. On the other, several different thalassaemia mutations might be regularly found in each chromosome type, indicating no relationship between these haplotypes and specific defects.

Coupling of specific β -thalassaemia defects to specific haplotypes

In a first attempt to characterize specific mutations in β -thalassaemia, we examined randomly chosen DNA samples from patients clinically homozygous for β^0 -thalassaemia. β -Globin genes were cloned with their flanking sequences as a 7.5-kb *Hind*III fragment in bacteriophage Charon 28 (ref. 18), as previously described⁷. Surprisingly, our first three β -gene clones (one of Greek and two of Italian origin) all showed the same nucleotide substitution, within codon 39, yielding a translation stop signal⁶⁻⁹ (see Table 1). Examination of the polymorphic restriction sites in the β -gene cluster of these three patients and their parents revealed linkage of the nonsense mutation genes with haplotype II chromosomes.

The coupling of one haplotype with a specific thalassaemia mutation suggested that each haplotype might indicate the presence of a specific mutant allele. Isolation and characterization of β -globin genes from thalassaemia DNAs representing the various haplotypes in our panel might then provide an efficient, systematic approach to the complete molecular description of the disorder in this population. We chose DNA samples that would permit rapid, unequivocal coupling of haplotypes with DNA mutations within β -genes. We did this by selecting patients homozygous for specific haplotypes, when available, or those heterozygous at the *Ava*II site located in the second intervening sequence (IVS-2)¹⁶⁻¹⁹ of the β -gene. In the latter case, by analysing bacteriophage clones containing the β -genes, we deduced which of the two β -genes had been cloned, even though the cloned region did not contain any of the other polymorphic sites used to define the haplotypes.

Further striking evidence of coupling of haplotypes and specific mutant genes was obtained for two other previously

described β -thalassaemia genes. First, three β -gene clones arising from haplotype I chromosomes contained the G $\rightarrow$ A substitution within IVS-1^{10,11} that leads to aberrant processing of precursor β -RNA²⁰ and a β^+ phenotype (Table 1). Second, a gene isolated from a haplotype III chromosome of a Turkish individual revealed a G $\rightarrow$ A transition at the splice junction of IVS-2 (Table 1), a mutation that prevents normal RNA processing (R. H. Treisman, N. Proudfoot, M. Shander and T. Maniatis, in preparation) and yields a β^0 phenotype¹². Because this mutation alters a cleavage site for the restriction enzyme *Hph*I normally found at the IVS-2 splice junction^{12,19}, we analysed our DNA samples by restriction mapping with this enzyme. All of six other chromosomes of the haplotype III pattern tested demonstrated the abnormal gene map after *Hph*I digestion,

Table 1 Summary of DNA sequences of β -thalassaemia genes

Haplotype	Thalassaemic mutation	Other sequence changes
I	IVS-1 (G) ...TTAGTCTATTTTCCACCTTAGGCTG...	31 Leu None
I	Exon-2 38 39 40 Gln (C) Arg ...ACCTAGAGG... Stop	None
III	IVS-2 splice junction 103 104 Phe Arg (G) ...TTCAGGATGAG...	G $\rightarrow$ T (position 74, IVS-2)
IV	Exon-1 7 9 Glu (A) Ser ...GAG--GTCT...	C $\rightarrow$ T (codon 2) C $\rightarrow$ G (position 16, IVS-2) G $\rightarrow$ T (position 74, IVS-2) C $\rightarrow$ T (position 81, IVS-2) T $\rightarrow$ C (position 666, IVS-2)
V	IVS-1 splice junction 29 Gly (G) ...GGCAGATTG...	G $\rightarrow$ T (position 74, IVS-2)
VI	IVS-1 consensus 29 30 Gly Arg (T) ...GGCAGGTTGGCATC...	C $\rightarrow$ T (codon 2) C $\rightarrow$ G (position 16, IVS-2) G $\rightarrow$ T (position 74, IVS-2) C $\rightarrow$ T (position 81, IVS-2) T $\rightarrow$ C (position 666, IVS-2)
VII	IVS-2 (internally) 745 (C) ...CAGGTACCAT...	C $\rightarrow$ T (codon 2) C $\rightarrow$ G (position 16, IVS-2) G $\rightarrow$ T (position 74, IVS-2) C $\rightarrow$ T (position 81, IVS-2) T $\rightarrow$ C (position 666, IVS-2) C $\rightarrow$ T (position 20, 5'-untranslated region)
VIII	5' flanking region -87 (C) ...CCACAGCTAGGGTTGGCCAATCT...	G $\rightarrow$ T (position 74, IVS-2)
IX	Same as II	None

β -Globin genes were cloned in 7.5-kb *Hind*III restriction fragments of genomic DNA in bacteriophage Charon 28 (ref. 18). Genomic DNA was digested to completion with *Hind*III and centrifuged in a 10-47% sucrose gradient in a Beckman SW27 rotor at 27,000g for 23 h. DNA of ~7.5 kb was ligated to calf alkaline phosphatase-treated *Hind*III arms of Charon 28 DNA and packaged *in vitro* into viable phage³⁰. Phage were screened by the Benton-Davis procedure³¹ using probe prepared by nick-translation from a plasmid containing the *Bam*HI-*Eco*RI fragment encompassing IVS-2 of the normal β -gene (provided by Arthur Nienhuis). The cloned β -globin genes of positive recombinants were subcloned as 4.4-kb *Pst*I fragments in pBR322 for DNA sequence analysis. All DNA sequencing was performed by the method of Maxam and Gilbert³². Fragments were labelled at their 5' or 3' termini and either strand-separated or subjected to secondary digests before sequencing. We analysed 13 independent β -globin genes of 10 unrelated β -thalassaemic patients. Genes of haplotypes V-VIII were sequenced completely; those of haplotypes I-IV and IX were extensively sequenced, except for the central 500 bp of IVS-2. Representative examples of genes of haplotypes I-III have been sequenced completely by others (refs. 8, 10, 11 and R. H. Treisman *et al.*, in preparation). Normal sequences are shown in parentheses above the putative thalassaemic mutations. Haplotype VI-VIII genes are separated from the others to indicate that the proposed mutations require further supportive evidence from functional studies (see text).

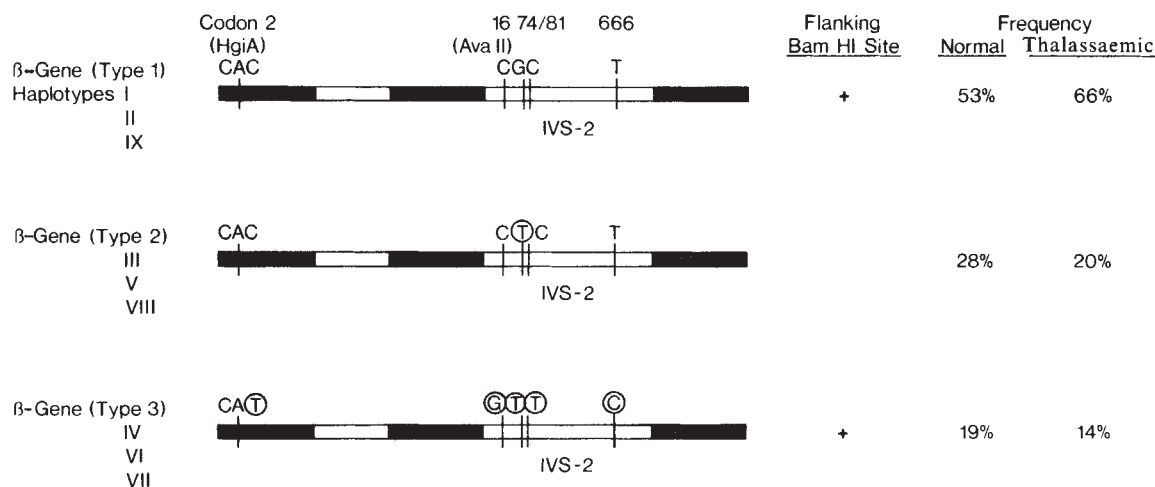


Fig. 2 β -Globin gene frameworks (gene polymorphisms). Schematic representation of polymorphic DNA sequences detected in β -globin gene clones (see Table 1). Except for the single nucleotide substitutions that lead to thalassaemia, the β -genes of haplotypes I, II and IX are identical in sequence to the normal gene sequenced by Lawn *et al.*¹⁹. Nucleotide sequence differences circled in the type 2 and type 3 genes are polymorphisms on the basis of their presence in multiple β -globin genes with different primary defects (see Table 1) and by the presence of all five changes of the type 3 gene in a cloned *AvaII*(-) gene of a non-thalassaemic Greek individual. Type 3 genes differ from type 1 and type 2 genes in lacking two intragenic restriction sites: the CAC→CAT change in codon 2 obliterates an *HgiA* I site and the C→G substitution 16 nucleotides into IVS-2 alters an *AvaII* site¹⁶.

whereas 34 of 36 β -thalassaemia chromosomes of various other haplotypes did not (see Table 2 and below). Therefore, this specific mutation was strongly coupled to haplotype III chromosomes on the basis of both gene cloning and restriction mapping.

In view of the coupling of three specific β -thalassaemia genes with haplotypes I, II and III, we analysed single genes representing the remaining haplotypes to elucidate the spectrum of mutant genes in this population. As indicated below, when possible, we assessed by restriction mapping the coupling of additional, new mutant genes with haplotypes.

DNA sequences of cloned β -genes

We examined the DNA sequence of 13 independent β -globin genes of 10 unrelated β -thalassaemia individuals, and a normal gene with the *AvaII* restriction polymorphism in IVS-2. Each previously undescribed mutant gene has been entirely sequenced. It is noteworthy that eight different β -thalassaemia genes were represented in the clones encompassing all nine haplotypes in our panel. Genes isolated from haplotypes IV–VIII revealed new mutations (see Table 1). Several conclusions regarding normal β -gene sequences and new putative thalassaemia mutations are derived from these data.

β -Globin gene polymorphisms: When compared with the normal gene sequence reported by Lawn *et al.*¹⁹, nucleotide substitutions that were common among different β -thalassaemia genes identified five polymorphisms of the β -sequence: a C→T substitution within codon 2, and C→G, G→T, C→T and T→C changes in IVS-2 positions 16, 74, 81 and 666, respectively. That these five changes are true polymorphisms was demonstrated by examining a normal gene of the *AvaII*(-) variety.

Further consideration of these polymorphisms indicated the existence of three β -globin gene frameworks into which various thalassaemia mutations have been introduced (Fig. 2 and Table 1). Type 1 is characteristic of β -genes of haplotypes I, II and IX and contains no DNA polymorphisms. Type 2 genes have a single polymorphism, the G→T substitution at IVS-2 position 74, and include thalassaemic genes of haplotypes III, V and VIII. Type 3 genes, encompassing haplotypes IV, VI and VII,

display all five polymorphisms. These various gene frameworks can be identified in the Mediterranean population by restriction mapping in that type 2 genes are linked to a *Bam*HI polymorphism¹⁵ 3' to the β -gene (see Fig. 1 and Table 1) and type 3 genes are recognized by the IVS-2 *AvaII* polymorphism. By restriction mapping, we estimate that type 1, 2 and 3 genes, respectively, represent 53, 28 and 19% of β^A -genes and 66, 20 and 14% of β -thalassaemia genes in the Mediterranean population (Fig. 2).

Putative thalassaemia mutations: From their DNA sequences, we have identified eight different β -thalassaemia alleles (Table 1), four of which have been reported previously. The molecular defects of genes isolated from haplotypes I–IV are either evident from the nucleotide sequence itself^{7–9} or established by gene expression in heterologous systems (ref. 20 and R. H. Treisman, N. Proudfoot, M. Shander and T. Maniatis, in preparation). Of the new mutant genes isolated here, that present in haplotype V, which contains a G→A substitution within the IVS-1 splice junction, is obviously a mutant of RNA processing, analogous to the IVS-2 splice junction mutant (R. H. Treisman *et al.*, in preparation). The two genes derived from haplotype VI and VII chromosomes are also possible mutants of β -RNA processing. In particular, the single substitution within the conserved consensus sequence²¹ of the IVS-1 splice junction in the gene of haplotype VI (Table 1) may have an adverse effect on either the rate or fidelity of processing of the RNA precursor. The C→G substitution at IVS-2 position 745 in the gene of haplotype VII (Table 1) generates a new CAGGT sequence that could well serve as a new (and deleterious) internal splice site²¹ in the β -RNA precursor. The accumulation of large amounts of β -RNA precursor containing IVS-2 sequences in the erythroid cells of a β^+ -thalassaemia individual with this gene is consistent with this nucleotide change (T.-C. Cheng and H.H.K., unpublished observations).

The gene isolated from haplotype VIII is a novel β -thalassaemia allele that is an excellent candidate for the first naturally occurring mutant of gene transcription in a mammalian system. Except for the IVS-2 position 74 polymorphism, its sequence is identical to that of the normal gene reported by Lawn *et al.*¹⁹. Upstream from the start of the β -gene at position -87, however, a single substitution (C→G) is observed in the vicinity of the CCAAT homology²², a region which seems

Table 2 Linkage of haplotypes with β -thalassaemia mutations analysed by restriction enzyme analysis

Defect and associated haplotype	Restriction enzyme used	β -thalassaemia alleles of the specified haplotype that were positive/no. tested	β -thalassaemia alleles of other haplotypes that were positive/no. tested	β^A -alleles that were positive/no. tested
IVS-2 splice (III)	<i>HphI</i>	7/7	2/36	0/27
IVS-2 position 745(VII)	<i>RsaI</i>	5/6	0/49	0/42
-87 substitution (VIII)	<i>AvrII</i>	1/1	0/6	0/16

The presence of the specific defects was assessed by restriction mapping. For analysis of the IVS-2 splice junction mutation, the labelled probe was an *EcoRI*-*Bam*HI fragment that spans IVS-2: the normal β -gene fragment is 1.1 kb and the mutant, 1.4 kb. DNA digested with *RsaI* was analysed with β -cDNA³³ as probe. The creation of a new *RsaI* site by the IVS-2 position 745 substitution reduces the length of a normal 2.2-kb fragment to 1.9 kb. A 5' β -gene probe, extending from the extragenic *HpaI* site to the intragenic *Bam*HI site, was used to analyse DNAs doubly digested with *AvrII* and *Hind*III. In this case, the normal 5' β -fragment is 2.3 kb, while the mutant band is ~4 kb. Values are the number of alleles that were positive for the thalassaemia defect as detected by the restriction enzyme, divided by the number of alleles tested, either within a haplotype, outside it, or in the normal Mediterranean population.

Table 3 Gene incidence based on polymorphism haplotype

Haplotype	Thalassaemia defect identified	No. with defect/no. tested	Total no. with haplotype		
			Italians	Greeks	Turks
I	IVS-1 β^+	4/4	14 (31%)	28 (67%)	—
II	Nonsense codon 39	3/3	11 (24%)	3 (7%)	—
III	5' IVS-2 splice	7/7	3 (7%)	2 (5%)	2
IV	Frameshift codon 8	1/1	—	—	1
V	5' IVS-1 splice	1/1	—	—	—
	5' IVS-2 splice	2/9	4 (9%)	7 (17%)	—
VI	IVS-1 consensus substitution	1/1	5 (11%)	1 (2%)	—
VII	IVS-2 position 745	5/6	5 (11%)	1 (2%)	—
VIII	-87 substitution	1/1	1 (2%)	—	—
IX	Nonsense codon 39	1/1	2 (5%)	—	1
		26/34	45 (100%)	42 (100%)	4
Total = 91 genes					

The presence of specific defects was assessed by gene cloning and DNA sequencing (Table 1) and by restriction enzyme digestions using *HphI*, *RsaI* and *AvrII* (see Table 2 legend). Although we have cloned and sequenced only 3 genes of haplotype I, the β^+ -thalassaemia patient studied by Spritz *et al.*¹⁰ (whose DNA was given to us by B. G. Forget) was homozygous for this haplotype and is scored as one allele here. Of 34 β -thalassaemia genes that were analysed directly, 26 have been assigned a specific mutation. Within haplotype V, *HphI* digestion excluded the IVS-2 splice junction mutation in seven genes. If direct detection of the IVS-1 splice junction mutation in uncloned DNA were possible, as many as 33 β -thalassaemia genes out of 34 assayed might have been linked to a specific defect.

important in the efficient initiation of gene transcription^{23,24}. Functional studies of these new thalassaemia genes are in progress to establish unequivocally whether the various nucleotide changes result in the β -thalassaemia phenotype.

Although the mutations resulting in thalassaemia in the genes of haplotypes VII and VIII have not been rigorously established by functional criteria, additional restriction mapping data provide further support for the particular mutant assignments and also for coupling of these defects with their respective haplotypes. The IVS-2 position 745 substitution in the haplotype VII gene creates a new *RsaI* restriction site (GTAC)²⁵. Restriction mapping of both thalassaemic and normal DNAs with *RsaI* has been used to investigate whether this nucleotide change is a normal polymorphism and whether it is limited to haplotype VII thalassaemia chromosomes. As shown in Table 2, an abnormal *RsaI* map was found for five of six genes present in haplotype VII chromosomes, but in no other normal (β^A -) or β -thalassaemia genes. The observed agreement suggests strongly that this single substitution is the primary mutation and is linked only to haplotype VII β -thalassaemia chromosomes. An additional nucleotide substitution within the 5' untranslated region of this gene (Table 1) is probably a DNA polymorphism that may subdivide framework 3 genes.

Similarly, the substitution upstream from the β -thalassaemia gene of haplotype VIII alters a normal *AvrII* restriction site. An abnormal *AvrII* restriction map was found in uncloned β -thalassaemia DNA of haplotype VIII but neither in β -thalassaemia genes of other haplotypes nor in β^A -DNA (Table 2).

Exceptions to simple coupling of haplotypes with specific defects

While the simplest situation would be the absolute linkage of each haplotype to a different mutation, we have found that this is not the case. We have observed two types of exception: (1) the association of a single mutation with more than one haplotype; and (2) the presence of more than one mutation within a single haplotype.

The codon 39 nonsense mutation was identified in a clone having the rare haplotype IX as well as in three clones having the common haplotype II (Table 1). The second type of exception was observed in genes of haplotype VII. As noted above, one of six genes of this haplotype had a normal *RsaI* restriction map, indicating the presence of another type of β -thalassaemia gene in this group. Study of the IVS-2 splice junction mutation by *HphI* restriction mapping revealed both types of exception. Although all of seven genes of haplotype III have this mutation, two of nine genes of haplotype V that were tested also showed this defect, whereas all of 27 β -thalassaemia genes of other haplotypes did not (Tables 2, 3). From these data, we suggest that the IVS-2 splice junction mutant comprises a minority of haplotype V β -thalassaemia genes, while the IVS-1 splice junction mutant constitutes the majority. Although the presence of the nonsense codon 39 and IVS-2 splice junction mutants in two different haplotypes is consistent with multiple, independent origins of these mutations, recombination between $\psi\beta_1$ and β -genes is a more likely alternative in view of the independent assortment of the 5' and 3' collections of restriction sites

in the β -gene cluster¹⁶. Only the finding of identical mutations in β -genes of different basic frameworks would provide clear-cut evidence for multiple origins of a specific mutation.

Frequency of specific mutations and genetic heterogeneity

By combining gene cloning with restriction analysis, we have determined the specific mutation in 26 β -thalassaemia genes (Table 3). Of 20 genes associated with the haplotypes I-III and VII, 19 displayed linkage of mutation to haplotype. These four haplotypes are associated with 81% of Greek and 73% of Italian β -thalassaemia alleles. This strong relationship allows us to estimate the incidence of the various alleles in these Mediterraneans, as shown in Table 3. Molecular heterogeneity of β -thalassaemia is so extensive that only a minority of patients are homozygous for any defect. 26 of 30 Italian and 11 of 22 Greek patients were genetic compounds and not homozygotes. Haplotypes for β -thalassaemia appear to associate randomly, as the expected numbers of genetic compounds were 81% and 48% for Italians and Greeks, respectively, while 86% and 50% were observed.

Conclusions

The β -thalassaemias are among the first human genetic diseases to be examined using new techniques of recombinant DNA analysis. Previous studies used essentially unselected samples for molecular cloning and gene analysis. Genes of affected individuals were generally chosen on the basis of a clinical phenotype, that is β^+ or β^0 -thalassaemia, with little attention being paid as to whether they were likely to differ. The existence of multiple restriction site polymorphisms within the β -globin gene cluster allows the detailed characterization of chromosome regions in which mutant β -globin genes reside. Taking advantage of these polymorphisms to subdivide a panel of β -thalassaemia genes, we have isolated eight different mutant genes among the nine different haplotypes represented in Mediterraneans. Repeated isolation of the same mutant gene was largely avoided by selecting β -genes on the basis of their associated haplotypes. Seven of these eight genes are present in Italians from various locales in Italy and six are present in Greeks. Our results suggest that only rare additional alleles are likely to be discovered among Mediterraneans in further studies.

The coupling of specific mutations and haplotypes is an example of linkage disequilibrium in which specific alleles become associated with neutral changes in the DNA. Disruption of these linkages could result from multiple origins of specific defects or DNA recombination (or mutation) that would alter the associated restriction site polymorphisms. As indicated above, we have found examples of discordance of single defects with haplotypes, which may have arisen by recombination.

When specimens of different racial backgrounds are examined, we find that the mutations identified in Mediterraneans are not present in chromosomes of the same haplotypes. For example, Asian Indians with haplotype III and VII chromosomes did not have the mutations recognized by restriction mapping with either *HphI* or *RsaI* (unpublished observations), although almost all samples from Mediterraneans did. Therefore, similar analyses of the kind described here may uncover new sets of mutant genes in other populations.

Our analysis of cloned genes delineates the nature of sequence polymorphism within the β -gene itself. Rather than observing randomly scattered sequence polymorphisms, we found three gene frameworks (Fig. 2) into which mutations were introduced. The presence of a common polymorphism (that at IVS-2 position 74) in frameworks 2 and 3 suggests that it may be possible to develop an evolutionary history of β -genes by analysing additional clones. The presence of these frameworks in β -thalassaemia genes, as well as coupling of specific mutations to haplotypes, suggests that the observed mutations are of relatively recent origin. We conclude that they arose after fixation of the haplotypes and β -gene frameworks.

Our data indicate that only 15 of 87 (~17%) mutant genes in Italians and Greeks can be detected directly by restriction enzyme analysis in uncloned DNA using *HphI*, *RsaI* or *AvrII* (Table 3). Thus, direct DNA analysis of β -thalassaemia mutations will have a small impact on prenatal diagnosis in Mediterraneans unless new enzymes are discovered that recognise the common defects. DNA polymorphism analysis, on the other hand, is highly useful for prenatal diagnosis when linkage analysis within a family can be carried out^{26,27}. Such linkage analysis is necessary because similar haplotype patterns are associated with normal and β -thalassaemic chromosomes. The situation in β -thalassaemia is in contrast to that in sickle cell anaemia which can now be detected directly in DNA using either *DdeI* or *MstII*^{28,29}.

The coupling of haplotypes and specific mutations of a single-copy locus within a defined population is probably a general finding as it reflects the origin of rare mutations in particular chromosomal backgrounds. We anticipate that the strategy used here will prove useful in characterizing genes associated with other inherited diseases and in further study of globin mutants. This approach should greatly facilitate the search for novel mutations among phenotypically similar individuals and the complete molecular description of a genetic disease.

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LETTERS TO NATURE

A new thermometer for external galaxies

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We introduce a new spectroscopic method at radio frequencies to measure the temperature of giant cloud complexes in external galaxies. We have detected the $\lambda = 1.3$ cm $J, K = 2, 2$ inversion transition of NH_3 in the nucleus of the galaxy IC342. This result, taken together with our previous detection of the $J, K = 1, 1$ transition¹ allows us to deduce a kinetic temperature of 50 ± 15 K and an equivalent size scale of at least 25 pc for the molecular gas. The results are consistent with line formation in the nucleus where very active star formation is taking place.

Microwave observations of the spectral lines of various molecules have been used with varying degrees of success to derive density, temperature, size scale and mass of clouds in the interstellar medium²⁻⁴. To infer gas kinetic temperatures for molecular clouds within our own Galaxy, the lower rotational transitions ($J = 1-0$, $J = 2-1$, $J = 3-2$) of CO and the inversion transitions of the lower metastable states ($J = K$) of NH_3 have proven to be most reliable. The CO transitions (mainly $J = 1-0$ observations) appear to be optically thick and to be in thermodynamic equilibrium with H_2 through collisions. Thus, the brightness temperature of the line reflects the kinetic temperature (T_K) of the molecular cloud. The technique with NH_3 utilizes the fact that the metastable states are coupled to each other principally through collisions with H_2 because the radiative lifetimes between the K -ladders are very slow. Therefore one can derive from the ratios of population in several nearby metastable states a rotational temperature (T_R) which in fact closely approximates T_K . Most commonly, the $J, K = 1, 1$ and $2, 2$ inversion transitions are used with the assumption that these lines are approximately optically thin, as indicated by the relative intensities of hyperfine structures. As these two metastable doublets are close in frequency, their excitation will be similar. The ratio of antenna temperature is then (see ref. 5, for basic formulae)

$$\frac{T_A(2, 2)}{T_A(1, 1)} \cong \frac{\tau(2, 2)}{\tau(1, 1)} \cong \frac{\nu_{2,2} |\mu_{2,2}|^2 N_{2,2}}{\nu_{1,1} |\mu_{1,1}|^2 N_{1,1}} \\ \cong \frac{20}{9} f e^{-41.5/T_R}$$

$$N_{JK} \propto g_{JK} (2J+1) \exp(-h(BJ(J+1) + (A-B)K^2)/kT_R) \\ |\mu_{JK}|^2 = K^2 \mu_0^2 / J(J+1)$$

where τ is the optical depth, N is the column density, μ_0 is the dipole moment, g_{JK} is a statistical factor accounting for the nuclear spin of the hydrogens ($g_{JK} = 1$ for $K \neq 3n$, $g_{JK} = 2$ for $K = 3n$), A and B the rotational constants, and f accounts for whether or not the hyperfine structure is resolved ($f = 1$ if unresolved).

Observationally, the ratio of the $J, K = 1, 1$ and $2, 2$ line strengths is quite sensitive to temperature in the range 5–80 K. The empirical results show that the ratio of $T_A(1, 1)/T_A(2, 2)$ is reliable in distinguishing between regions of various temperatures. For example, $T_A(1, 1)/T_A(2, 2) \cong 1$ for H II region-large molecular clouds ($T_K = 30$ –70 K), and $T_A(1, 1)/T_A(2, 2) \cong 10$ for dark clouds ($T_K = 10$ K). Additionally, these two transitions (at $\nu_0 = 23.7$ GHz) are separated by only 28 MHz in frequency so that they can be measured simultaneously with the same receiver and the same spectrometer. Thus, their ratio

is well determined independent of the absolute calibration of the spectra.

A comparison of the observational results of these two techniques using CO and NH_3 is shown in Fig. 1 for various types of interstellar clouds in the Galaxy. The correlation between the two methods indicates that these independent techniques are both sampling the gas temperatures. The advantage of the NH_3 observational method is that it circumvents the following difficulties, especially in extragalactic studies. First, beam dilution: the CO observations must spatially resolve the source to determine the brightness temperature reliably. In the Galaxy, interstellar CO observations generally resolve the source. However, for the external galaxies, the CO data suffer from an indeterminate amount of beam dilution. In contrast, the analysis based on NH_3 data depends on the ratio of line strengths. Because the NH_3 optical depths are in general low and the excitation for the two lines are comparable, any beam dilution effects are cancelled out in taking a ratio. Second, self absorption: several CO lines in galactic sources exhibit self absorption features due to temperature and/or density gradients in the cloud combined with the high opacity of the CO transition. Again because of the low opacities of the NH_3 lines, self-absorption effects are not observed and are not likely to be important. For the external galaxies, we therefore recognize that studies of the NH_3 molecule represent an important new tool.

Improvements in sensitivity have allowed radio astronomers to begin to use molecular line observations to study the external galaxies⁶. CO exhibits the strongest lines and has been the most fruitful molecule for study so far. For the galaxies where the CO line is most intense, the emission has been partially mapped and serves as a tracer of the H_2 density. Thus, in combination with H I data, the total hydrogen distribution can be studied in galaxies. However, without *a priori* knowledge of the spatial

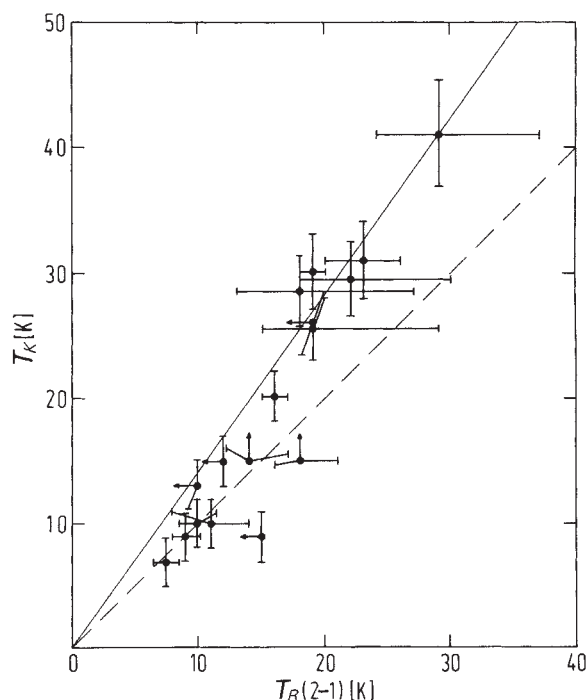


Fig. 1 A comparison of $T_R(2-1)$ as deduced from NH_3 and T_K as deduced from CO for various sources in the Galaxy^{8,9}. The dashed line is $T_R(2-1) = T_K$ and the solid line is $T_R(2-1) = 0.7 T_K$ (empirical results for compact H II regions⁸). The correlation demonstrates the reliability of temperatures derived through NH_3 measurements as compared with CO measurements. The exact coefficient is uncertain but theoretical calculations indicate that in general $T_R(2-1)$ will be less than T_K .

structures, CO cannot be used to measure gas temperatures in this instance due to the severe beam dilution. The detection of extragalactic NH_3 through its two lowest metastable transitions therefore constitutes indeed a new thermometer for giant gas complexes in external galaxies.

Using the MPIFR 100-m telescope, we have now detected the $J, K = 2, 2$ inversion transition of NH_3 in the nucleus of the ScD galaxy IC342, in addition to our previous detection of the $J, K = 1, 1$ transition¹. We can thus derive the first reliable measure of the kinetic temperature for extragalactic molecular gas. The observations were made using a cooled parametric amplifier frontend ($T_{\text{sys}} = 200$ K) and a 256×312.5 kHz filter-bank spectrometer. Both the $J, K = 1, 1$ and $2, 2$ lines fall within the passband of the spectrometer. We detect a main beam brightness temperature of 0.032 ± 0.004 K for the $J, K = 1, 1$ transition and 0.030 ± 0.004 K for the $2, 2$ transition. Taking into account the errors in the measurements and the uncertainty in the conversion from T_R to T_K , we estimate the kinetic temperature to be 35–70 K. A detailed analysis of these results is given elsewhere⁷; we briefly summarize our findings as follows. From the detected brightness temperatures, we infer that the emission must cover an equivalent area with a diameter of

at least 25 pc. From excitation arguments, we derive excitation temperatures and hence densities. We then estimate the total mass as a function of size scale. Together with estimates based on the column density and abundance arguments, we arrive at a total mass detectable via the NH_3 lines of the order of $10^7 M_\odot$. Such large and warm gas complexes imply a large cooling rate due to emission in atomic and molecular lines. A possible heating mechanism would be warm dust grains which must first be heated by direct star light. We therefore infer the presence of numerous young OB stars. Detailed calculations indicate that 10^3 – 10^5 OB stars would be required in the nuclear region of IC342. We conclude that the detection of the NH_3 lines, indicating the presence of warm gas, implies very active star formation in the core of this spiral galaxy. In fact, the star formation rate may be 10 times higher than within the nuclear region of our own Galaxy.

In spite of its weak lines, NH_3 may be the most promising molecule, for investigations of the physical conditions of molecular clouds in galaxies.

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Observing interplanetary disturbances from the ground

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The scintillation of celestial radio sources due to small-scale turbulence along lines of sight through the interplanetary medium provides a convenient, ground-based method of monitoring disturbances in interplanetary space. With the sensitive 3.6-hectare Array at Cambridge we have carried out a new programme in which ~900 sources were observed each day for more than 1 yr. When the long-term average scintillation behaviour of each source had been accurately determined we found that transient disturbances could be clearly distinguished. We detected large clouds of enhanced turbulence moving out from the Sun and were able to track them to distances beyond the Earth's orbit. Our observations differ from recent work elsewhere¹ in that we used a larger grid of sources and observed them more continuously over a wider range of solar elongations. To illustrate the information that is now being obtained we present observations for 10–21 December 1978, a period of considerable solar activity for which spacecraft and other data are also available.

The measurements were carried out at 81.5 MHz with the 3.6-hectare Array which enables sources to be observed simultaneously at meridian transit over a wide range of declinations². Intensity scintillation in the band 0.1–3 Hz was detected, averaged and sampled at intervals of 10 s on to magnetic tapes. The noise fluctuation per sample was ~0.5 Jy and each source was observed for ~120 s. Data contaminated by interference, solar noise or ionospheric scintillation were removed by a two-stage interactive program² and the r.m.s. scintillating flux density, ΔS , was then found for the transit of each source.

To detect interplanetary space disturbances it was first necessary to determine the average systematic dependence of ΔS on solar elongation, ϵ , for each source as accurately as possible. Previous work³ had shown that the variation of ΔS with ϵ depended on the angular size of the source, rising more

steeply near the Sun for smaller sources, but measurements over 8 yr (during 1967–74) revealed no detectable solar cycle variations, nor has any significant ecliptic latitude dependence been found⁴. 250 strongly-scintillating sources were selected and ~400 daily observations of each were used to plot individual $\Delta S(\epsilon)$ functions; smooth curves were fitted through the points by means of Chebyshev polynomials, thereby avoid-

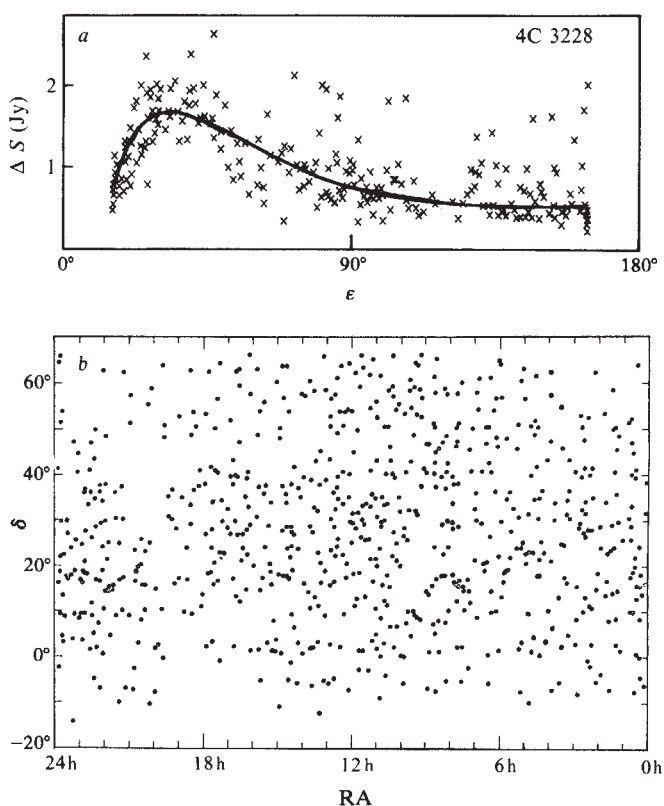


Fig. 1 *a*, Daily values of the r.m.s. scintillating flux density, ΔS , plotted against solar elongation, ϵ , for a typical source. *b*, The grid of radio sources observed.

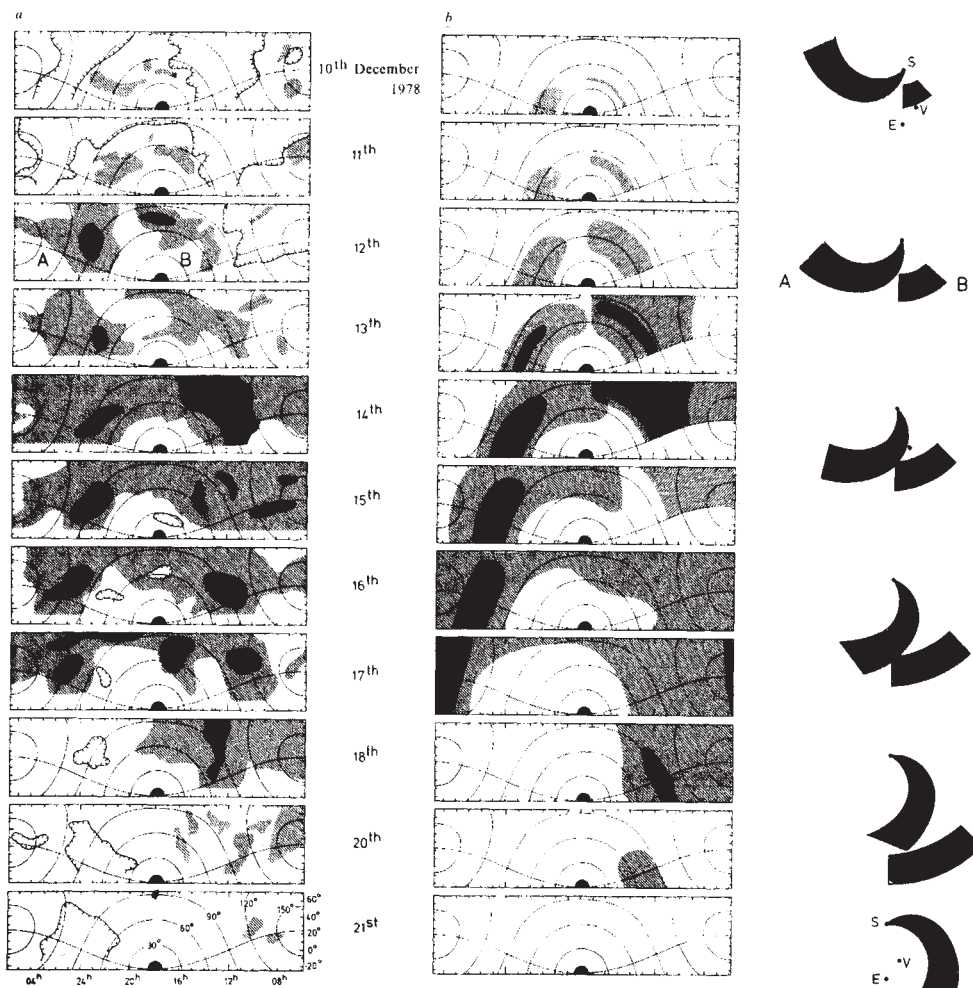


Fig. 2 *a*, Maps of the disturbance factor, g , across the sky. Light shading $1.25 < g < 1.5$; dark shading $g > 1.5$. The fenced contour lines denote $g < 0.5$. The Sun is shown as a 15° diameter disk. *b*, Theoretical maps computed for the model illustrated in *c*. *c*, Schematic diagram of the assumed model. See also Fig. 3*b*. S, V, E denote the Sun, Venus and Earth.

ing possible errors due to averaging over intervals of finite elongation. The sources were next sorted into groups according to the value of the ratio $\Delta S(\max)/\Delta S(90^\circ)$ derived from the smoothed curves. Having thus separated the sources according to their angular size, daily points for the sources in each group were combined, after scaling to the same mean value of ΔS , to provide smoothed $\Delta S(\epsilon)$ curves. In this way we obtained a set of standard $\Delta S(\epsilon)$ curves against which to compare the day-to-day values of the full grid of more than 900 sources. Our method of normalization avoided the necessity of assuming some particular model of the solar plasma, although the $\Delta S(\epsilon)$ curves were very similar to those derived from such a model⁴. A typical $\Delta S(\epsilon)$ curve through the day-to-day points for a source is shown in Fig. 1*a*.

Interplanetary disturbances were detected by calculating daily values of the disturbance factor, $g = \Delta S/\Delta S(\epsilon)$, for each source in the grid shown in Fig. 1*b*. Maps illustrating the variation of g across the sky are shown in Fig. 2*a*, where approximate contours have been fitted by eye to the data. The maps are centred on the position of the Sun so that the scale of right ascension, indicated on the frame for 21 December, shifts from day to day. Note that data for 19 December are not available. The observed declination range is -10° to $+70^\circ$, but the frames extend to declination -20° to include the Sun, enlarged to 15° diameter, on the ecliptic. To aid visual interpretation, lines of constant solar elongation at 30° intervals are also shown. Note that the maps are not instantaneous images of the sky. The measurements take 24 h, from west to east, so that the appearance of rapidly-moving features may be distorted, as in a camera with a focal plane shutter.

Features are included on the maps only when we believe them to be real. For example, on 16 December, the small patch of decreased scintillation at $\epsilon \sim 60^\circ$ due north of the Sun shows a well-correlated variation on all 13 sources within this zone.

When the edges of features take simple shapes, such as the boundary of the enhancement at $\epsilon \sim 90^\circ$ on the ecliptic west of the Sun on 17 December, they can be located to an accuracy of $\pm 5^\circ$. The extent to which further details have been blurred may be assessed from (1) the relatively coarse contour intervals adopted in this preliminary study and (2) the fact that wiggles in the contours have been drawn only when they embrace correlated variations on at least five sources.

To interpret the maps in terms of three-dimensional structures in space, we first consider the constraints imposed by the theory of propagation through random media. *In situ* measurements on spacecraft^{5,6} show typical plasma density enhancements of two to three times the mean density which last for about 1–3 days. On the assumption, to be justified later, that the small-scale turbulence responsible for scintillation increases in direct proportion to the large-scale plasma density, we now consider the effect of some simple structures consistent with spacecraft data.

Figure 3*a* shows the location of the important scattering zones along any line of sight. The diagram refers to a particular model³ of the solar plasma but is mainly controlled by the inverse-square dependence of density with solar distance. Spherical symmetry has been assumed, for which there is good evidence over heliocentric latitudes up to 60° (ref. 4). Within a cone defined by $\epsilon \leq 45^\circ$ no enhanced scintillation is expected because this is the zone where scintillation saturates due to the onset of strong scattering and blurring of the diffraction pattern by sources of finite diameter. It is this which causes $\Delta S(\epsilon)$ to decrease for $\epsilon < 30^\circ$, as shown in Fig. 1*a*. The broken line in Fig. 3*a* indicates the limit beyond which any disturbance of twice the average density would give $g < 1.25$ and hence escape detection in our present data reduction.

Consider first a single cloud, about 0.5 AU in diameter when it reaches 1 AU, in which the density is twice the mean density

at any solar distance. Travelling at typical solar-wind speeds the cloud would cover a distance of 1 AU in about 4 days so that on successive days it might appear as shown in Fig. 3a. For lines of travel at angles $\phi \geq 90^\circ$ to the Sun-Earth direction, the cloud would be undetectable, except perhaps for about 1 day along lines of sight such that $\varepsilon \sim 45-60^\circ$. For $\phi < 90^\circ$ the cloud would be detectable for about 5 days. The largest enhancements of scintillation would be seen at $\varepsilon \geq 90^\circ$ when the cloud best fills the main scattering zone, which occurs for $\phi < 45^\circ$. To produce a disturbance lasting longer than about 5 days, the Sun must eject a succession of clouds or a continuous stream. This would take the form of a co-rotating sector, due to solar rotation, as sketched in Fig. 3a. A long-lived enhanced density disturbance of this type would be seen first to the east of the Sun and then to the west, and would be detectable for 10-12 days in all.

We found that the above constraints enabled the general form of the three-dimensional structure of the observed enhanced scintillation zones to be determined with little ambiguity. Figure 2a shows a persistent enhancement to the east (labelled A) at $\varepsilon \sim 90-150^\circ$ which lasted for 8 days. This can be explained only by the approach of a co-rotating stream. The sector must cover about $30-45^\circ$ in heliocentric longitude to account for the wide range of elongation over which it was seen on 12-13 December, and its position in longitude can be determined to $\sim \pm 5^\circ$ from the location of the sharp trailing edge, seen at $\varepsilon \sim 60-90^\circ$ near the ecliptic during 15-17 December. The extent of the sector in heliocentric latitude, which can be gauged from the rapidly increasing northward ecliptic latitude observed on 10-12 December, must be at least 45° .

The co-rotating disturbance cannot account for the enhancement (labelled B) seen above the Sun on 10-11 December and

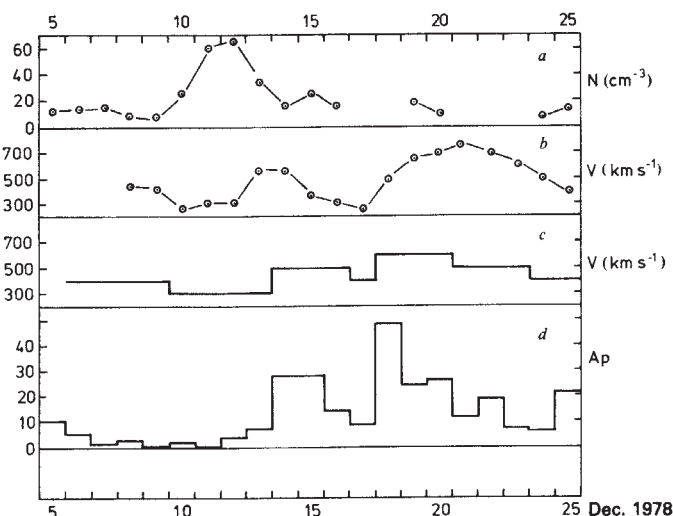


Fig. 4 *a*, Daily values of the solar plasma density observed near Venus. *b*, Daily values of the solar wind speed near Venus at about noon UT. *c*, Daily average solar wind speed near Earth. *d*, Daily average magnetic indices, A_p .

expanding over a wide range of ecliptic latitude on 12-13 December. A and B remain clearly separated on the maps until 14 December, and the co-rotating feature discussed above could not possibly give rise to enhanced scintillation on the ecliptic at $\varepsilon \sim 90^\circ$ to the west on 13 December. Disturbance B was extended roughly along lines of constant elongation and was most prominent at $\varepsilon \sim 75-120^\circ$ to the west on 14 December over ecliptic latitudes -20° to $+90^\circ$. These characteristics indicate a tangentially extended disturbance passing Earth to the west and cutting off near the Sun-Earth line. The radial thickness of the shell must have been ~ 0.5 AU at a distance of 1 AU to explain the range of elongation of the enhancement on 12-13 December. Its radial velocity, derived from the outward motion of the leading edge, was $350 \pm 50 \text{ km s}^{-1}$, and the shell must have been about to engulf the Earth on 12 December. The notable increase of the enhancement on 14 December, which we interpret as due to the tangential alignment of the line of sight with the shell, indicates a spread in heliocentric longitude of at least 45° , and a corresponding extent in heliocentric latitude above the ecliptic.

To confirm these semi-quantitative estimates, we adopted the simple model shown in Fig. 3b and assumed that within each disturbance the density was doubled. The shell covered 50° in longitude and latitude above the ecliptic, and travelled at 350 km s^{-1} . The spiral envelope of the co-rotating sector corresponded to a velocity of 430 km s^{-1} and the sector covered latitudes $\pm 50^\circ$ and 45° in longitude. In this simple analysis we ignored any interaction between the disturbances, such as might have occurred after 14 December, and computed the scintillation adopting weighting functions along the line of sight mentioned earlier. The contributions of each disturbance may be distinguished in the theoretical maps shown in Fig. 2b. Note that the shell has passed from view after 15 December. Our model simulates the observations remarkably well, considering its simplicity, and we do not believe that it could be significantly modified. Details such as the sphericity of B are not accurately known.

Before comparing our conclusions with other space and geophysical data we return to our assumption that enhanced scintillation correlates with enhanced plasma density. Much evidence now exists that enhancements of scintillation may be identified with regions of compressed plasma moving outwards from the Sun in advance of high-velocity zones. The compression regions usually occur between fast and slow solar wind streams^{7,8} or, less often, near the leading edges of disturbances associated with solar flares^{9,10}. In some cases there is evidence

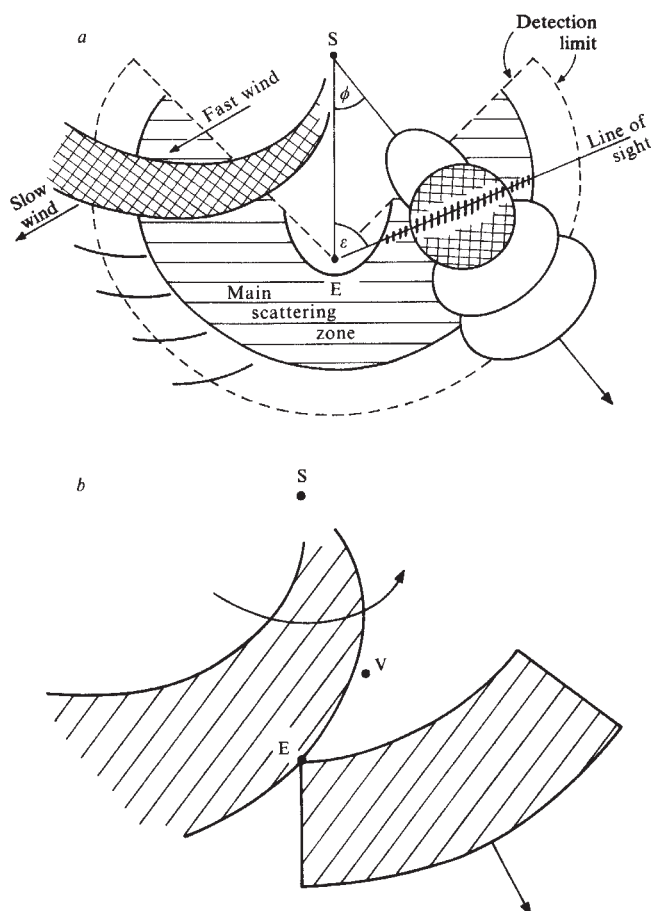


Fig. 3 *a*, Schematic diagram showing the location of the dominant scattering zones along any line of sight. *b*, The configuration of the model disturbances on 14 December.

that scintillation enhancements may be displaced slightly from the zone of maximum density¹¹.

Relating our present observations to previous work, we infer that the high-velocity stream generating co-rotating disturbance A must arrive at the Earth around 17–18 December, following the departure of the compression region. The observation of a zone of decreased scintillation to the east during 18–21 December supports this conclusion, as the plasma density within fast solar wind streams has less than average density⁵. Disturbance B, which clears the Earth around 14 December, should also be followed by a zone of increased velocity.

Daily average values of spacecraft measurements and associated geomagnetic data¹² are presented in Fig. 4, together with solar plasma measurements near Venus by the Pioneer-Venus Orbiter¹³. All these observations are in excellent agreement with our model, providing further confirmation that enhanced scintillation relates to increased plasma density. A fast solar wind stream arrived at the Earth on 18 December and initiated strong geomagnetic activity. This stream was associated with a coronal hole of positive polarity which appeared on the Sun about five rotations earlier¹². Another high-velocity zone of shorter duration reached the Earth on 14 December and again caused a geomagnetic disturbance. Finally, we note that our model predicts the arrival of enhanced density at Venus on 10 December, and its departure on 12–13 December. The observed density follows this pattern, and the high-velocity zone reached Venus on 13 December, about 1 day before reaching the Earth. A shock wave at Venus was observed at 07.50 UT on 13 December, and at 01.27 UT on 14 December at the Earth¹³. This shock has been tentatively associated^{13,14} with solar flares commencing either at 23.32 UT on 10 December in McMath plage region 15697 or at 18.32 UT on 11 December in region 15694. As the wind speed rose to 500 km s⁻¹ within a few hours after the arrival of the shock at Venus, and as the shock corresponded closely with the inner boundary of disturbance B, we assume that these are related events. It is evident, however, that disturbance B could not have been generated by either of the flares because its leading edge had already advanced beyond 0.5 AU from the Sun by 12.00 UT on 10 December.

Our results demonstrate the potential of scintillation as a method of studying the morphology of interplanetary disturbances in three dimensions. The ability to track disturbances in the range 0.5–1.5 AU should be useful in the field of solar-terrestrial relationships, and as a technique for predicting geomagnetic storms. The forecasting of magnetic activity depends, of course, on the early recognition of disturbances that will later engulf the Earth. In the present case we believe that the salient features of disturbance B, including the likelihood that it would hit the Earth, were evident by about 12 December, giving 2 days warning of the magnetic storm on 14 December. Similarly for disturbance A, the co-rotating nature became clear by 13 December, giving 5 days warning of the storm on 18 December.

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Wave-produced bubbles observed by side-scan sonar

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When a wave breaks in deep water, forming a whitecap, many small bubbles are generated and carried below the surface by turbulence. The breaking wave forms a strong acoustic target when viewed from below by an inverted side-scan sonar. The bubbles remaining in the water after the breaking wave has passed out of the sonar beam are also good acoustic scatterers and their drift towards or away from the sonar provides a means of remotely measuring that component of the near-surface current without interference of the flow and without exposing instruments to the hazard of violent near-surface conditions. This current changes rapidly as the wind changes. The sonar display shows regions of convergence or divergence of bubble clouds, perhaps associated with Langmuir circulations, and may, in calm weather, respond to the presence of surface slicks.

We have used an inverted telesounder¹, a modified side-scan sonar, to probe the sea surface and near-surface scatterers. The sonar consists of a single linear 248 kHz transducer mounted horizontally a few centimetres above a reflecting plate inclined upwards from the horizontal at about 20° (Fig. 1). Pulses of sound 0.08 ms (12 cm) long are transmitted at 0.25 s intervals in a vertical plane in a narrow beam which is modulated by interference with sound reflected from the plate. The pattern of the effective beam at the surface consists of a series of ovals extending away from the sonar, typically about 1 m wide and with length increasing from 1 to ~5 m.

The sonar was mounted by divers on a frame resting on the sea bed in Loch Linnhe, north-west Scotland, at a depth of 34 m and some 700 m from shore, to which it was connected by cable (see ref. 2 for details of earlier experiments and site). Recording was on a conventional graphic recorder displaying time versus the time delay between transmission of sound and reflected signal or effectively, for uniform sound speed, time versus range of scatterers. The beam was directed towards 290° T (true north) and, during the period of observation, the wind and swell were approximately from 225° T.

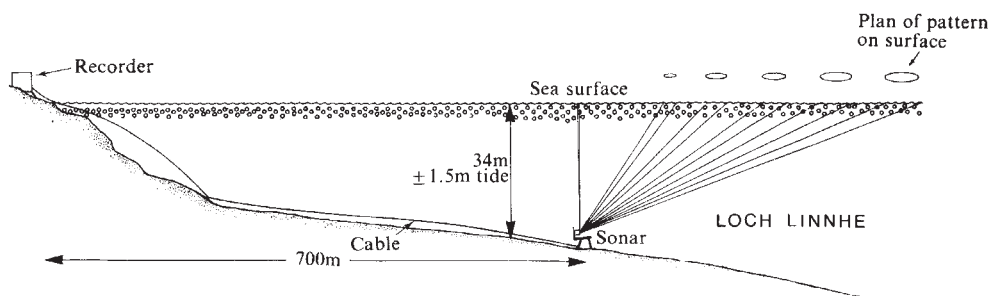
Figure 2a shows the display in conditions of low wind speed (figure times are given in Fig. 3). The return from the sea surface directly above the sonar is shown by the distinct lower dark band; in the original record both swell and wind waves can be distinguished, with periods of about 8 and 2.5 s respectively. Above the surface (at greater range from the transducer) appear a series of bands corresponding to sound scattered from the ovals on the sea surface. Their mean position is in reasonable accordance with the prediction of sound intensity shown on the right of Fig. 2a (see ref. 1 for theory). Undulations can be seen which correlate with the swell in the direct surface return. The fading of the record at the beginning and the end may be due to slicks which reduce the surface capillary waves and hence the effective scattering at 6 mm sound wavelength.

In windy conditions, the bands are overlain by parallel streaks which trend towards, or away from (as in Fig. 2b), the sonar and last for 1–5 min. Figure 2c covers a period of rapid change in pattern during a squall whilst Fig. 2d shows a more gradual change in streak direction. The streaks often start with a sharp dark line, indicating a strong scatterer moving at about 1.7 m s⁻¹ in these examples towards the sonar. Occasionally two such

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Fig. 1 Diagram of the telesounder installed in the sea in Loch Linnhe, near Oban, north-west Scotland.



lines are present (B, Fig. 2d). The streaks occasionally continue between bands (see, for example, Fig. 2b) indicating that the scatterers are not all in the surface ovals (as in Fig. 2a) but below the surface at shorter ranges.

The speed with which the scatterers producing the streaks move parallel to the sea surface towards the sonar is plotted in Fig. 3, together with wind speed and direction, and tidal variation (measured by the sonar). The streak speed is much less than that of phase speed of the waves (about 12.5 m s^{-1} for swell and 3.9 m s^{-1} for wind waves) although the lines commencing the streaks have speeds approximately equal to the component of the wind wave phase speeds in the direction of the sonar beam, which, like the wind, were always directed towards the sonar in the examples shown here. We deduce that the lines are the breaking crests of waves that produce trailing clouds of bubbles which, left in the water, are then advected by the near-surface current to form the streaks.

These bubbles have been described elsewhere^{3,4}. Most bubbles have radii of $20\text{--}150 \mu\text{m}$, have rise speeds of $<1 \text{ cm s}^{-1}$ and are concentrated near the surface². Their scattering cross-section decreases exponentially below the surface in vertical distances of $0.4\text{--}1.0 \text{ m}$ in winds of $4\text{--}12 \text{ m s}^{-1}$. At a horizontal

range of 60 m their rise speed would contribute $<0.5 \text{ cm s}^{-1}$ to the current away from the transducer, which is within the accuracy to which the bubble drift can be determined from the sonar display ($\sim 1 \text{ cm s}^{-1}$). The speeds shown in Fig. 3 may thus be identified, to within the experimental error, as the component of the near-surface current towards the transducer. The current varies rapidly with change in wind speed (Fig. 2c). At wind speeds of about 7 m s^{-1} (Fig. 2b) the waves break so frequently that most of the near-surface water contains bubbles which produce detectable scattering. No obvious regular patterns (like, for example, cloud streets) can be seen and the bubble population at a particular location appears to be determined principally by the earlier presence of breaking waves and advection. Clouds are typically $1\text{--}2 \text{ m}$ wide and little lateral diffusion is apparent. Indeed, most of the streaks become gradually narrower with time, which suggests that the reduction in acoustic scattering cross-section of the clouds, due to bubbles returning to the surface or dissolving, occurs more rapidly than their lateral spread by turbulent diffusion. Small-scale convergences and divergences of the bubble streaks are, however, visible (for example at A, Fig. 2b), and these may be associated with Langmuir circulation patterns⁵.

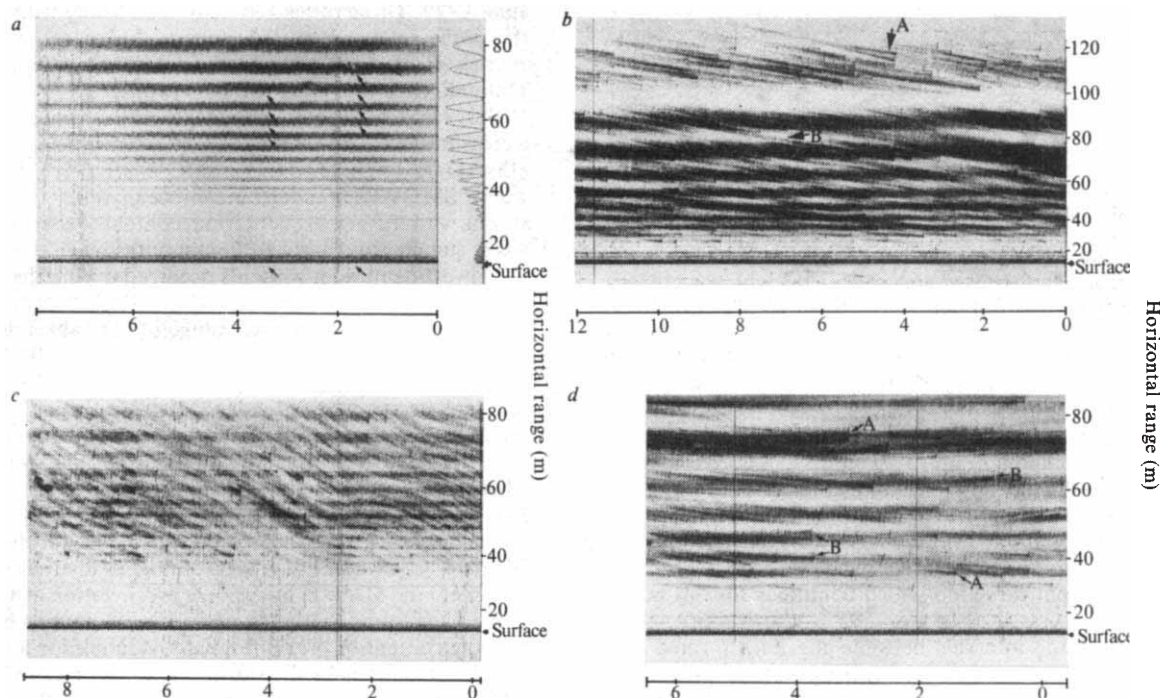


Fig. 2 Sonar displays. Note that the time increases to the left and that the non-linear horizontal distance along the surface is shown at the right. *a*, Low wind speeds. The arrows indicate groups of swell. The calculated intensity of a return from a uniform field of reflectors at the surface is shown at the right. The band of marks 2 min after the start of the record is due to an unknown noise source. *b*, The scatterers, identified as clouds of bubbles producing the streaks in the bands, are moving away from the transducer. Converging scatterers are shown at A whilst the scatterers at B are between the surface scattering bands and are therefore sub-surface. *c*, A squall showing a rapid variation in streak pattern. Compare with *a* and note the presence of (sub-surface) scatterers (bubbles) between the surface bands, especially at 4 min and 50 m range. *d*, A shift in current producing a change from approaching to receding streak pattern. Note the single line (breaking wave crest) commencing the streaks at A and double lines at B, where two waves in succession have broken within the sonar beam.

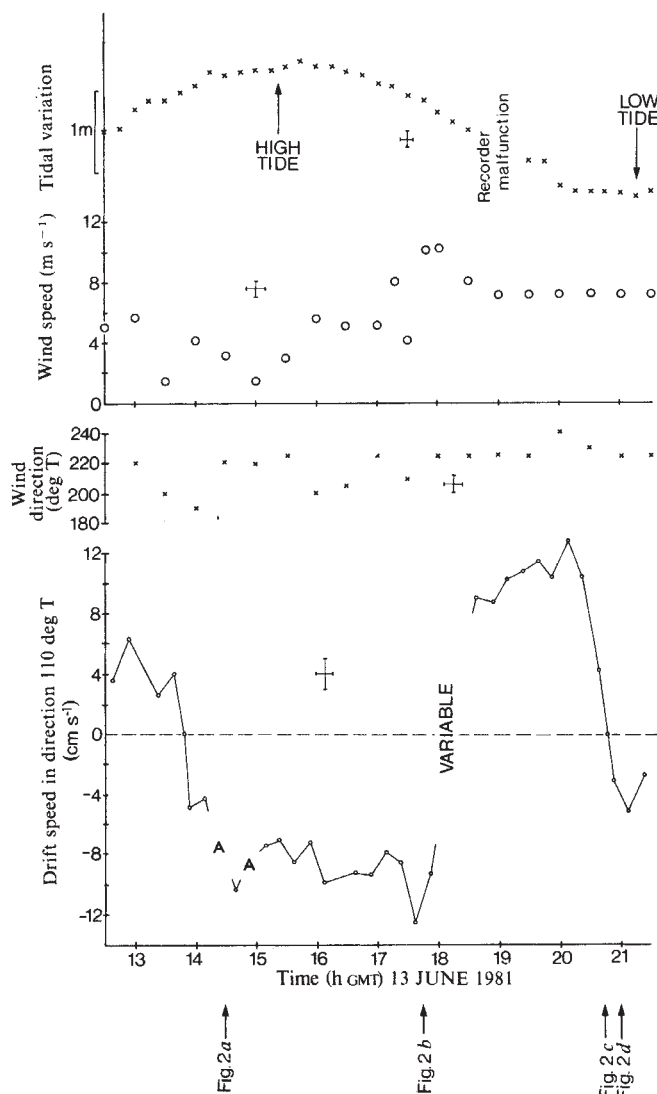


Fig. 3 The variation of tidal variation (measured from the sonar relative to an arbitrary zero), wind speed and direction (recorded at the laboratory of the Scottish Marine Biological Association, Dunstaffnage, ~1.1 km south-east of the sonar) and the 15-min average drift speed of scatterers in direction 110° T. The times corresponding to Figs 2a–d are noted on the time axis. At some times (marked A; for example, the period shown in Fig. 2a) no streak lines can be seen because the wind is not strong enough to produce breaking waves and bubbles which can be detected by the sonar.

Much of Fig. 3 can be explained in terms of wind and tide. The positive component up to about 1330 h is consistent with both wind and tidal flow. A southwesterly tidal flow should develop 1 h 50 min before high tide⁶ and indeed appears as a negative component at 1345 h slightly delayed, perhaps by the opposing wind. The increasing wind at 1745 h produces a change in current direction which continues for 3 h but then decays.

The mean time intervals between the lines formed by the breaking crests decrease with increasing wind speed and are in accordance with earlier observations of bubble clouds⁷. Wind waves are, however, seen to break more frequently than bubble clouds appear to be generated, and it appears that only the more vigorously breaking waves may be detected by the present sonar.

The method of determining near-surface currents by remote acoustic sensing may have particular value in severe wave conditions when the signal-to-noise ratio is enhanced because more bubbles are produced and when conventional moored instruments are prone to considerable hazard and error due to

mooring motion. The technique may be useful in measuring the sea state and frequency of wave breaking, as well as perhaps monitoring slicks or coastal fronts and internal waves which modulate the near-surface currents.

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Storm trajectories in eastern US D/H isotopic composition of precipitation

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The isotopic content of precipitation falling at a point provides information of potential importance concerning atmospheric circulation and climate change^{1–6}. The isotopic content of precipitation, however, is controlled by the particular meteorological processes acting at a given time and place and is subject to considerable variation. We have measured the deuterium-to-hydrogen ratios (D/H) of precipitation at Mohonk Lake, New York, from individual storms over a 2-yr period, July 1977–June 1979. These ratios varied systematically with the locus of the paths followed by the storms: the more seawards (and southerly) the paths and the colder the temperatures at Mohonk, the lower were the D/H ratios.

Very large differences in the D/H ratios from storm to storm were observed. The δD range for all storms was -7% to -170% $\delta D = (D/H \text{ sample} - D/H \text{ SMOW}) / (D/H \text{ SMOW}) \times 10^3$, where SMOW is standard mean ocean water. In general the storms with the highest deuterium contents occurred during the warm summer and early autumn months while the storms with the lowest deuterium contents occurred during the cold winter months.

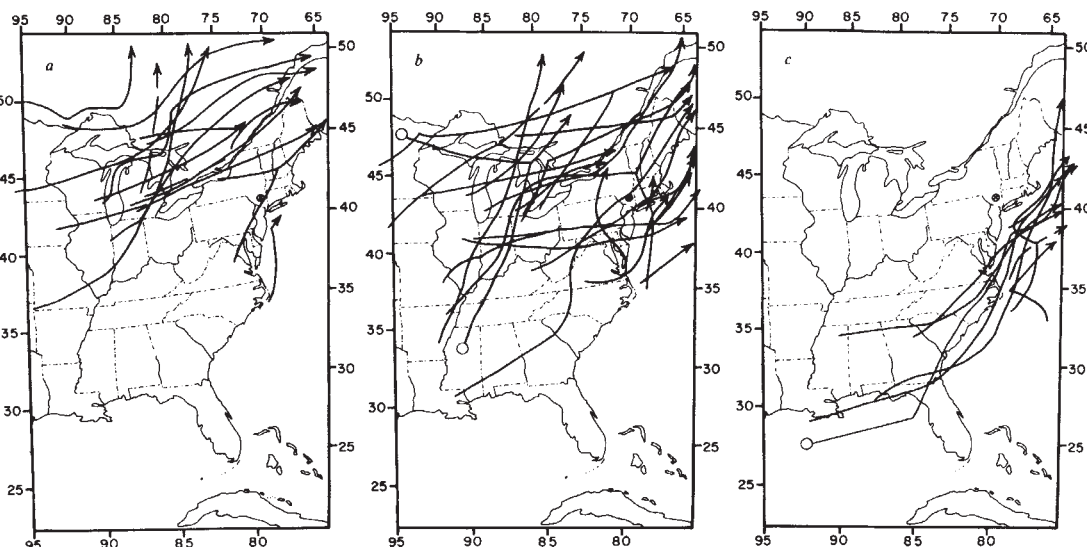
There was a large to small range of δD values during single storms. During two storms on 20–21 January 1978 and 25–26 January 1978, precipitation was also sampled sequentially at a separate location in Palisades, New York. For the storm of 20–21 January, the δD for the first 2 inches of snow was -177% , while the ice crust formed by a mixture of the later snow with freezing rain that fell at the end of the storm had a δD of -75% . For the storm of 25–26 January 1978, the δD for the first $\frac{1}{2}$ inch of rain was -78% compared with -54% for the last $\frac{1}{4}$ inch. We detail elsewhere⁷ how meteorological analysis can be used to explain the δD ratio of cyclonic precipitation at a given time.

We have found that a strong relationship exists between the isotopic content of precipitation at Mohonk for a given storm and the path taken by the storm (the storm track or trajectory): in general the δD decreases systematically as the locus of the storm trajectories is displaced seawards (Fig. 1).

Storms in the eastern US are frequently produced as a result of the interaction of cool, dry continental air with warm, humid maritime air. There is often a rather narrow and sharp transition zone, known as the polar front, that separates these two distinct air masses. Cyclones occur more frequently along the polar front.

During the colder half of the year when the area of polar influence expands and the meridional temperature gradient is

Fig. 1 The trajectories of the low pressure centres of storms occurring between July 1977 and June 1979. The trajectories were separated into five groups on the basis of the δD of the precipitation that fell at Mohonk Lake near New Paltz, New York. Three of the groups are shown: a, $\delta D \geq -30\%$; b, $\delta D = -61$ to -90% ; c, $\delta D \leq -121\%$. The two intermediate groups (isotopic ranges: -31% to -60% , and -91% to -120%) not shown have trajectories intermediate to those shown. $\otimes$, The location of Mohonk Lake. $\circ$, The origins of two storms of January 1978 from which a sequence of samples was taken at Palisades, New York.



larger, the polar front is typically located further south and is more often well defined. Accordingly during the winter both cyclone frequency and intensity are greater and the average position of the paths taken by the cyclones are located further south. For the eastern US this means a greater percentage of storm tracks with coastal or offshore positions during the winter.

Similarly, during the height of the ice ages, the area of polar influence was greatly expanded and the average position of the storm tracks was much further south than today⁸. Therefore a greater percentage of the ice age storms would have been classified as coastal storms and would have produced precipitation at New York and on the North American ice cap with correspondingly low values of δD .

There is a straightforward explanation for this relationship between the δD of precipitation and position of the storm tracks. Air undergoing condensation from cooling due to lifting experiences a progressive depletion of the heavy isotopes^{9,10}. Thus the precipitation from a given air parcel that forms last, or at greatest altitudes, invariably has lower δD and $\delta^{18}O$ values than any earlier precipitation from that air parcel¹. When a frontal surface is aloft, most of the precipitation forms in the air above the front. It follows then that the higher the frontal surface, the lower the δD of precipitation tends to be. In general the higher the frontal surface above a particular location, the further south the storm is from that point. Storms that stay far to the south or remain well seawards of New York will tend to have the lowest δD because the frontal surface is always found at considerable altitude.

These observations lead us to predict a coherent spatial distribution of δD values around a typical extratropical cyclone. The D/H ratios should be highest for the precipitation in the warm sector of the storm, although they may be subject to some variations because much of the precipitation in the warm sector can be convective. The steep slope of the cold front should cause a dramatic decrease of the δD to the west of the surface position of the cold front (such behaviour has been described elsewhere¹¹). Northwards of the warm front there should be a coherent but gradual decrease of δD consistent with the gentler slope of the warm front.

Such a synoptic pattern of δD values can be used to account for the rather wide range of storm track positions for those storms producing intermediate δD values (as in Fig. 1b). Two storms may have very similar tracks but quite different δD values if the positions of the warm fronts are different. This is illustrated in Fig. 2, in which four separate storms together with their storm tracks are shown. When storm tracks are either far north or far south of Mohonk, there is little ambiguity about the δD values. However, for storms with intermediate tracks, the position of the warm front is critical for determining δD .

Figure 2a shows the storm of 9 January 1978; the track lay far north of Mohonk Lake and, as is characteristic for such

Table 1 δD of precipitation from storms

July 1977 to June 1978			July 1978 to June 1979		
Date	Amount (inches)	δD (%)	Date	Amount (inches)	δD (%)
6 July	0.27	-35 ± 2	3-4 July	1.12	-84 ± 5
8 July	0.17	-21 ± 2	10 July	0.33	-40 ± 2
17 July	0.17	-12 ± 1	17-18 July	0.33	-38 ± 1
19 July	0.20	-25 ± 1	24 July	0.24	-28 ± 1
25 July	0.70	-33 ± 1	28-29 July	0.29	-20 ± 1
1-2 August	0.12	-22	31 July-1 August	0.38	-56 ± 3
3 August	0.17	-44	4 August	0.20	-45 ± 2
5 August	0.18	-35	6-7 August	1.33	-38 ± 2
12 August	0.27	-14	11-12 August	0.34	-44 ± 2
17 August	0.41	-26	24-25 August	0.75	-7 ± 1
22 August	0.17	-39	28 August	0.18	-17 ± 2
31 August	0.16	-29	31 August	1.62	-45 ± 1
13-14 September	0.53	-18	12 September	0.31	-24 ± 2
16-17 September	1.27	-46	18-19 September	2.31	-35 ± 1
20-21 September	1.78	-50	22-23 September	0.25	-12
23-26 September	4.10	-65	4 October	0.22	-25
1-2 October	2.26	-43	6-7 October	0.70	-29
8-9 October	1.32	-11	25-27 October	0.40	-22
14-15 October	0.59	-21	7-8 November	0.16	-37
16-17 October	1.00	-14	17-18 November	1.12	-31
19-20 October	0.51	-47	23-24 November	0.68	-67
7-9 November	5.18	-44	29-30 November	0.30	-161
25-26 November	1.22	-88	3-4 December	0.42	-83
29 November-1 December	1.02	-62	8-10 December	1.42	-84
9 December	0.21	-66	21-22 December	0.95	-92
12-15 December	1.49	-40	24-25 December	1.51	-112
18-19 December	0.51	-146	1-4 January	2.79	-62
20-21 December	0.89	-58	5-6 January	0.18	-170
25 December	0.32	-73	7-8 January	2.30	-89
1-2 January	0.31	-113	12-14 January	0.50	-81
6-9 January	2.50	-48	17-18 January	0.25	-99
13-14 January	1.20	-116	20-21 January	2.91	-87
17-18 January	1.38	-96	24-26 January	2.18	-86
19-20 January	0.89	-145	7-8 February	0.32	-127
25-26 January	1.56	-75 ± 1	19 February	0.24	-170
6-7 February	0.76	-138	23-25 February	1.70	-69
3 March	0.32	-123	25-27 February	1.00	-109
14 March	1.31	-30	2 March	0.18	-72
16 March	0.16	-130	6-7 March	1.33	-45
25-27 March	2.38	-55	10-11 March	0.60	-110
19-20 April	1.25	-55 ± 2	14 March	0.10	-39
5-6 May	0.80	-134 ± 2	24-25 March	0.82	-46
9 May	0.80	-50 ± 2	29 March	0.22	-55
10 May	0.75	-34 ± 1	2-3 April	0.27	-30
14-15 May	1.97	-31 ± 2	4-5 April	0.24	-66
16-18 May	1.91	-67 ± 2	8-10 April	1.27	-64
24-25 May	2.34	-57 ± 2	14-15 April	1.10	-63
31 May	0.40	-22 ± 1	26-28 April	1.38	-37
3 June	0.72	-51 ± 4	3-4 May	0.22	-29
7-8 June	0.92	-21 ± 4	12-14 May	0.59	-42
9 June	0.45	-62 ± 2	18-20 May	0.26	-50
13 June	0.16	-38 ± 2	23-25 May	4.30	-53
21 June	0.72	-21 ± 4	26 May	0.26	-48
			5 June	1.01	-46
			11 June	1.74	-41
			18 June	0.36	-21
			30 June	0.21	-20

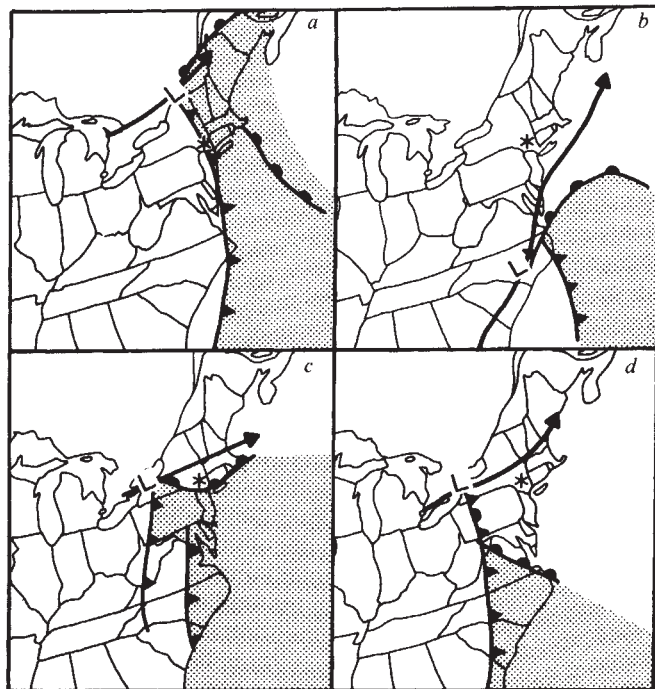


Fig. 2 the δD values are given for four different storms: a, 9 January 1978, 12.00 GMT, $\delta D = -48$; b, 20 January 1978, 1.00 GMT, $\delta D = -145$; c, 15 December 1977, 00.00 GMT, $\delta D = -40$; d, 2 January 1978, 00.00 GMT, $\delta D = -113$. The shaded region shows the warm sector of the storms. Heavy arrows show storm trajectories. *, The collection site at Mohonk Lake, New York.

storms, most of the precipitation at Mohonk occurred as rain in the warm sector or near the warm front. Such storms mostly produce a high δD .

This is to be contrasted with the storm of 20 January 1978 (Fig. 2b), which took a far more southerly track. Throughout this coastal storm, Mohonk Lake remained deeply embedded in the cold air. Characteristically, the precipitation fell almost entirely as snow (it was a major snow storm for the entire northeastern US) and δD was very low.

Figure 2c, d shows two storms with intermediate and almost identical tracks but with a large difference in the δD values for each storm which is a direct result of the difference between the positions of the warm fronts for each storm. The storm of 15 December 1977 (Fig. 2c) had a quite high value of δD . Most of the precipitation fell when the warm front lay only a short distance south of Mohonk. In contrast, the warm front for the storm of 2 January 1978 (Fig. 2d) remained well south of Mohonk through the time of the precipitation and consequently the δD value for this storm was much lower.

We have also found a significant relationship between the δD values and the mean effective temperature during the precipitation. The quantity T is calculated by weighted averaging of the temperature using precipitation amounts. Then, for January, storms with at least 0.75 inches of precipitation, we obtain

$$\delta D = -119.5 + 5.0 \bar{T} \quad (1)$$

where δD is the expected value of δD (in ‰) and T is in °C. The correlation coefficient is 0.91, which is surprisingly high considering that we are relating a surface parameter to a process occurring above ground level. For most large January storms the principal maritime source is the warm waters extending from the Gulf of Mexico through the Caribbean Sea to the Atlantic Ocean. Source conditions vary rather little from storm to storm but temperatures at New York may vary considerably. Apparently the temperature at New York during times of precipitation in January varies coherently with the frontal position and with the important precipitation producing processes aloft.

Two distinct results of the present findings have great potential value. First, the isotopic composition of the precipitation

from a given storm depends strongly on the details of the storm's path, structure and evolution. We have shown⁷ previously that by taking these details into account, we can calculate accurately the isotopic composition of precipitation falling at a given time and place. This shows that the δD values of precipitation or vapour in the air act as a tracer of atmospheric motions. The δD value depends most strongly on the vertical profile of the ascent rate of the air in the cloud which cannot be measured directly at present. The measurement of δD at a given time, therefore, provides a means for estimating the vertical profile of the ascent rate of the air in the clouds above.

Second, a climatic signal can be extracted from the δD values of precipitation from storms. Changes in atmospheric circulation redistribute the trajectories of storms (Fig. 1) and/or the amounts of precipitation (Table 1) in both time and space. An integrated sample of precipitation or a portion thereof provides the isotopic signal recorded in tree rings, freshwater fossils or glacial ice cores. Routine analysis of all storms would provide a better foundation for evaluating climate change from isotope studies.

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A 1.2-Myr record of isotopic changes in a late Pliocene Rift Lake, Ethiopia

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The Hadar fluvio-lacustrine formation (Ethiopia) contains an original hominid fossil *Australopithecus afarensis* and an abundant vertebrate faunal assemblage^{1,2}. The formation^{3,4} consists of a 150–300 m thick sequence of fine-to-coarse detrital sediments, originating from the Ethiopian Plateau escarpment, with some interbedded pyroclastic layers. The three lower members of the formation were formed in a lacustrine environment, which was occasionally interrupted by fluvial drainage. The upper member is quite distinct and formed as an alluvial fan delta. This sedimentary sequence was deposited at the end of the Pliocene between 3.9 and 2.7 Myr, as indicated by magnetostratigraphy⁵ (the palaeomagnetic record spans the Gilbert–Gauss transition period), K/Ar dating^{6,7} and biostratigraphy⁸. We consider here the oxygen and carbon isotope variations observed throughout the formation and their palaeo-hydrological implications.

Eighty-six carbonate horizons and 28 mollusc-bearing units were collected throughout the section, from the base to the top. The carbonates are mainly lacustrine nodules. However, at the base of the first member some micritic limestone beds occur and one horizon of the second member contains stromatolitic structures (oncolithes). These carbonates consist of a low magnesian calcite.

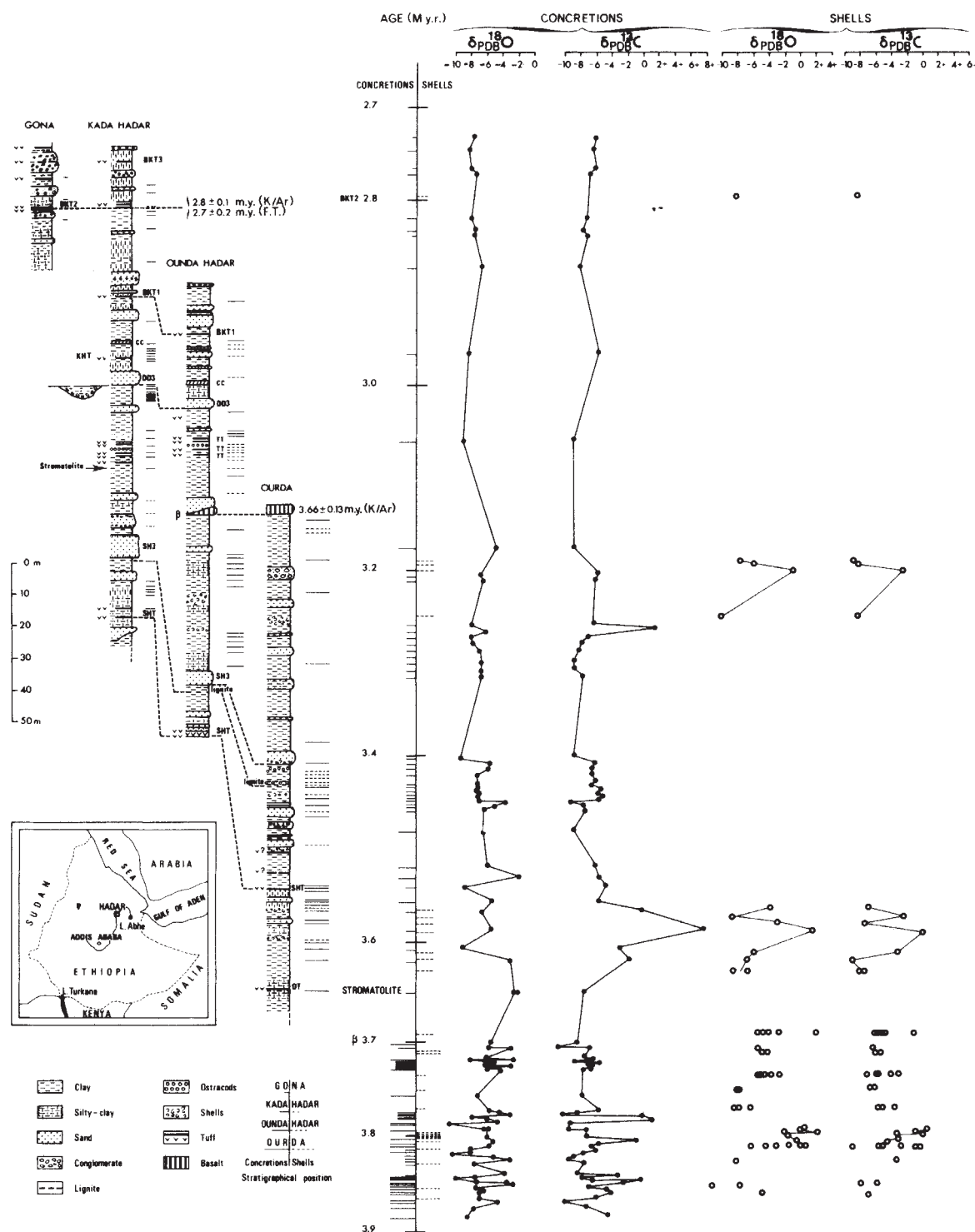


Fig. 1 The Hadar Formation. Lithology, chronology and isotopic changes in concretions and fossils.

The fossils which were collected are essentially shallow fresh-water gastropods (*Melania tuberculata*, *Bellamya* sp., *Cleopatra* sp.) and a bivalve (Unionidae). The shells are usually pure aragonite and do not show any evidence of post-depositional recrystallization.

The $\delta^{18}\text{O}_{\text{PDB}}$ values, from the base to the top of the section (Fig. 1), show large fluctuations between -10.8 and -2% for concretions and between -10.5 and 2.5% for shells. These values indicate highly variable palaeohydrological conditions during the episode, with phases of drastic evaporation inducing considerable enrichment of the water in heavy oxygen. The shells are systematically enriched in ^{18}O compared with the concretions. This enrichment reflects both the aragonitic nature of the shells which represents a 0.8% difference with calcite⁹

and the adaptation of molluscs to highly evaporated waters. For example, five species found within one single layer of the second member (Fig. 1) have $\delta^{18}\text{O}$ values ranging from -6 to 1% . Specific fractionation cannot explain such large differences, which are more realistically explained by environmental changes both in time and space, with conditions ranging from freshwater to highly evaporated and eventually brackish water. Most of these molluscs currently tolerate such conditions (see, for example, refs 10, 11 for Pleistocene Saharan lakes, or ref. 12 for rift lakes). That hydrological changes were very frequent in the Hadar lake, is shown by the ^{18}O variations in the stromatolitic structure (Fig. 2). It is difficult to assess any periodicity for the growth of oncolithes, but seasonal or annual cyclicity might be considered for those of biogenic origin^{13,14},

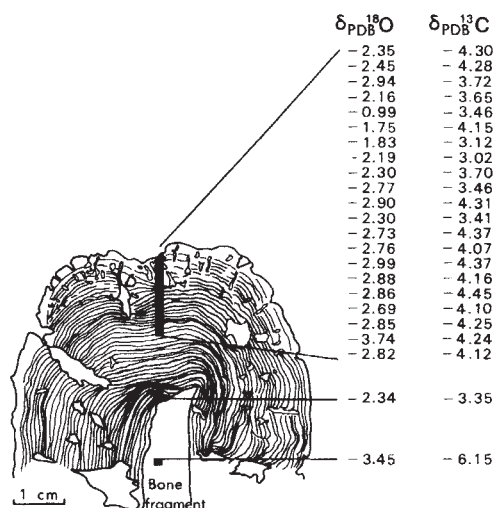


Fig. 2 Isotopic variations in one of the oncolites of the second member.

which seems to be the case here as indicated by the ^{13}C content (see below). Thus within a few years, changes as high as 3‰ (Fig. 2) occurred in the ^{18}O content of calcite, these should essentially reflect variations of the isotopic composition of the lake water. An increase in temperature is not likely to be responsible for a large negative shift of ^{18}O content in carbonates, because it would be largely counterbalanced by a positive effect associated with increased evaporation. Negative shifts of ^{18}O values are more realistically explained by influx from precipitations. Consequently, positive shifts, from the lowest $\delta^{18}\text{O}$ values to higher ones, must essentially be the result of evaporation periods which followed the precipitations. Thus variations in the ^{18}O content of carbonates can be related to contrasted hydrological regimes. Abundant precipitation induced sudden lake level rises; the isotopic composition of the water during such episodes was occasionally as low as $-12 \pm 1\%$ (SMOW), if we accept for calcite precipitation an average temperature $\sim 25 \pm 5^\circ\text{C}$, as suggested by faunal requirements¹⁵. Periods of high lake levels were rapidly followed by isotopic evolution typical of closed basins: the evaporation induced an enrichment of the water in ^{18}O , with $\delta^{18}\text{O}$ values as high as 0‰. Such rapid hydrological changes suggest that the lake was mainly fed by strong floods coming from the Ethiopian Plateau escarpment. This agrees with the facies distribution which also indicates rapid changes in the sedimentary dynamics.

Similar hydrological regimes were observed in nearby Holocene lakes by Fontes and Pouchan¹⁶ or Gasse *et al.*¹⁵. The $\delta^{18}\text{O}$ values of Lake Abhé water for the Holocene¹⁷ are close to our mean values, although the range of variation there (-11.9 to -5.8%) is not as large as it was in the Pliocene Hadar lake. The similar morphological situation in the Afar Rift, and the strong influence of runoff and drainage from the Ethiopian Plateau are common to both lakes. However, the higher $\delta^{18}\text{O}$ values which were occasionally present in the Hadar lake water suggest stronger evaporation phases at the end of the Pliocene leading occasionally to subaerial conditions. The occurrence of the flood plain facies which sporadically interrupt the lacustrine sequence of the three lower members supports this interpretation. The upper member, which is characterized by coarse alluvial fan deposits, does not show large isotopic changes. The relatively stable $\delta^{18}\text{O}$ values of carbonates (-8 to -6%) indicates a fast drainage system without any strong influence of evaporation. Thus the ^{18}O content of carbonates could reflect the average isotopic composition of the precipitation during this episode ($\delta^{18}\text{O} \approx -8.5\%$).

The ^{13}C content of carbonates (Fig. 1) suggests a noticeable contribution of biogenic CO_2 . A few concretions or shells show positive isotopic compositions for carbon, however, it is difficult to accept equilibrium with atmospheric carbon dioxide. Such positive $\delta^{13}\text{C}_{\text{PDB}}$ values are linked to sedimentological features

indicating a reducing environment (with lignite layers, for example), and thus to an unusual CO_2 metabolism¹⁸.

In conclusion, isotopic compositions of the Hadar lake carbonates do not reflect climatic conditions drastically different from those which prevailed during the Holocene in the area¹⁵. The lowest $\delta^{18}\text{O}$ values calculated for lake water indicate either a cold origin of the waters precipitated between 2,000 and 3,000 m on the Ethiopian Plateau, or a pronounced continental effect. A similar meteorological pattern of monsoon rains produced by cooling of humid air masses which could originate from the Gulf of Guinea, the Indian Ocean or exceptionally the Gulf of Aden, may have prevailed during the late Pliocene. The variability observed in the Hadar lake regime strongly suggests either a more contrasted seasonality as it has been proposed for the same epoch in Laetoli, Tanzania¹⁹, or longer term climatic oscillations, or both. The hydroclimatic evolution of the Afar Rift through time seems to differ from that proposed by Cerling *et al.*¹² for the Gregory Rift more to the south in Kenya. Their data in Lake Turkana support the hypothesis of a more humid period during late Pliocene. The inland situation of Lake Turkana could explain the difference between the two areas. However, the limited number of data presented and the various origins of the carbonates (shells, lacustrine and soil nodules) analysed in both areas preclude a detailed comparison of the two sites.

The palaeomagnetic data of the Hadar Formation have been computed again and calibrated with new radiometric dates⁶. The Cochiti events of the Gilbert period and the Kaena and Mammoth events of the Gauss normal period seem to be present in the Hadar sequence. T. J. Schmitt, A. E. N. Nairn, J. L. Aronson, R. C. Walter, M. Taieb, and J. J. Tiercelin are all involved in this new study.

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Bacteria accumulate silver during leaching of sulphide ore minerals

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Silver is extremely toxic to a wide range of bacteria¹⁻⁵, and has even been used in solution to control bacterial growth². In an investigation of the oxidation of several sulphide minerals containing traces of silver by a mixed culture of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*, it was noted that the bacteria accumulated silver. I report here that careful collection of most of the cells from such experiments yields a silver

concentrate, while the silver attached to the cells remaining in contact with leach residues is readily recovered by a conventional cyanidation technique. These results suggest that the bacterial leaching of valuable and non-valuable sulphide minerals in order to release atomically bound silver, and possibly gold, may be used as part of a recovery process for these elements.

Electron microscopic examination of bacteria collected from several experimental sulphide leaching systems, in which substrates contained silver, has shown that the bacteria involved in the leaching can accumulate silver in very large quantities and that their activity does not appear to be inhibited by silver contained in sulphide minerals. The manner in which silver is accumulated by the bacteria is illustrated in Fig. 1, which shows an electron micrograph of a single bacterial envelope prepared by washing a mixed culture of active bacteria collected from a sulphide leaching experiment with dilute KOH. The membrane is covered with small electron-dense particles of silver in the form of silver sulphide. These small Ag₂S granules have been observed to grow on the surface of bacteria collected at different stages of a batch leach finally accumulating to form large clumps. Cleaned suspensions of bacteria coated with such particles are very dark compared with normal bacterial suspensions and on drying at 100 °C, a shiny black deposit is produced. The amount of silver accumulated per gram of dry weight biomass varies in proportion to the silver content of the sulphide mineral being leached, but concentrations greater than 250 mg per g have been observed. The granules on the bacterial surface were analysed using a Philips EM 400 electron microscope microprobe analyser and were found to contain silver and sulphur in proportions suggesting the formulae Ag₂S. The granules are crystalline and results of polycrystalline electron diffraction indicate that the compound is the silver mineral acanthite (Ag₂S).

When initially observed on the surface of bacteria recovered from a sulphide leaching test, these small silver sulphide particles were considered to have been released from the matrix of the sulphide mineral being leached, and to have remained unoxidized. This hypothesis was supported by the fact that silver sulphide is unaffected by the action of acidophilic bacteria³. Further experimentation, however, has shown that these silver sulphide particles can be produced artificially on the surface of bacteria by adding silver in a soluble form to a suspension of bacteria which are actively oxidizing a sulphide mineral substrate containing no silver. The particles have also been observed forming on bacteria attached to the flat surface of silver-rich copper sulphide mineral (tetrahedrite) that had been introduced into a bacterial suspension growing on a sulphide mineral substrate free of silver. Attempts to obtain similar results with bacteria grown in the presence of ferrous sulphate alone have been unsuccessful but these silver sulphide particles

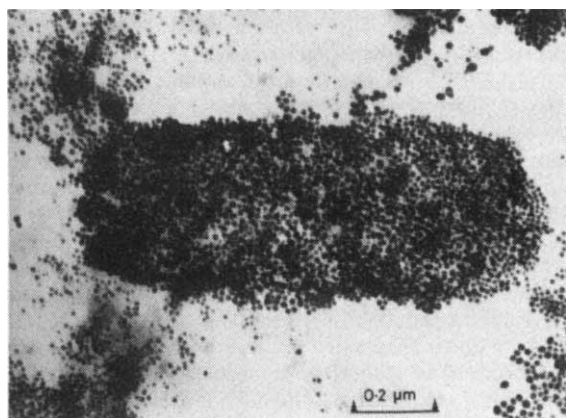


Fig. 1 Electron micrograph of a single bacterium emptied of its cell contents showing adhering silver sulphide particles, after recovery from a sulphide mineral leaching experiment in which the mineral contained trace quantities of silver.

can be formed on cells grown on elemental sulphur. Therefore, prerequisite for the formation of these particles is the presence of sulphide anions as well as silver cations. The reasons for the preferred nucleation of the silver sulphide on the bacterial wall are not clear but this preference is evident from the fact that in some experiments as much as 25% of the bacterial mass recovered after leaching of a silver sulphide mineral consisted of silver.

The finding reported here has interesting practical applications for the recovery of silver from sulphide ore minerals and is also of interest geologically, as it may indicate how silver was released, concentrated and relocated in surface ore deposits which previously had been affected by bacterial action.

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Mineralization of organic matter in the sea bed—the role of sulphate reduction

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The bacterial reduction of sulphate to sulphide at the sea bed is a key process in the oceanic sulphur cycle, and is responsible for the oxidation of organic matter which becomes buried below the oxic and sub-oxic zones of the sea bed. The oxic surface layer of the sea bed varies in thickness from a few millimetres in sheltered coastal areas to ≥ 1 m in pelagic sediments^{1,2}. Below this layer, organic matter is mineralized mainly by fermenting, denitrifying, sulphate-reducing and methane-producing bacteria. Sulphate reduction is the predominant terminal step in the mineralization processes of sulphate-rich shelf sediments where the sulphate reducers inhibit the methanogens by competing with them for common substrates^{3–5}. Sulphate reduction may therefore have a quantitatively important role in the overall oxidation of organic matter in the sea bed. Recently, concurrent measurements of oxygen uptake and sulphate reduction in a coastal sediment⁶ have demonstrated the importance of the sulphate-reducing bacteria in the mineralization of organic carbon. I present here the first comparative survey of aerobic and anaerobic mineralization in the sea bed based on direct rate measurements of the two processes. The results demonstrate a surprisingly high contribution from the sulphate-reducers. In coastal sediments, this specialized group of bacteria oxidized as much organic matter to CO₂ as did all the aerobic organisms. Their relative contribution decreased three fold over the continental shelf from the shore to a depth of 200 m.

Oxygen consumption and sulphate reduction were measured in shelf sediments using previously described techniques^{7,8}. Sediment cores were collected from 58 stations during cruises in Danish fjords and in Kattegat and Skagerrak, the two seas connecting the Baltic Sea with the North Sea. Measurements were made in the laboratory or onboard ship within 1 day after sampling. Sulphate reduction was analysed down to 15 cm depth in the sediment; measurements in cores of 2–3 m depth showed that most of the activity (75–95%) occurred in the topmost 0–15 cm (data not shown). Below 15 cm depth, I found significant (up to 40%) reduction only in cores from 200 m depth.

Figure 1 shows the data obtained from all the sediment stations. The sediment types consist of a wide range of grain

sizes (from medium sand to clay) and organic content (0.7–18% dry weight). There is a clear correlation between the measured rates of oxygen consumption and sulphate reduction in these shelf sediments. This is partly a result of their mutual regulation by the influx of organic matter to the sea bed through sedimentation from the water column^{9–13}. It is also due to the following inherent coupling between the two processes. During the bacterial reduction of sulphate, H_2S may be produced in large excess over the binding capacity of iron. The fraction of the H_2S which was precipitated by iron and converted into pyrite was calculated from the total (solid phase) reduced sulphur accumulation with depth and from the sedimentation rates (ref. 6 and B.B.J., unpublished results). The fraction varied from ~5% at 0–2 m depth to 10–20% at 20–200 m depth. Thus, an excess, corresponding to 80–95% of the H_2S produced, must have been re-oxidized back to sulphate at or near the sediment surface. The dark uptake of oxygen, which was the ultimate electron acceptor for the sulphide oxidation, therefore included 80–95% of the mineralization via sulphate.

This has important implications for the oxygen consumption of the coastal sea bed. The molar ratio of oxygen uptake to sulphate reduced averaged about 4:1 in sediments at 0–2 m and 2–20 m water depth (Fig. 1, broken line). As the oxidation capacity of 1 mol of sulphate is equivalent to 2 mol of oxygen, the ratio was 2:1 in terms of electron flow. Because 90–95% of the sulphide produced at 0–20 m depth was re-oxidized by oxygen, this means that ~50% of the oxygen uptake was

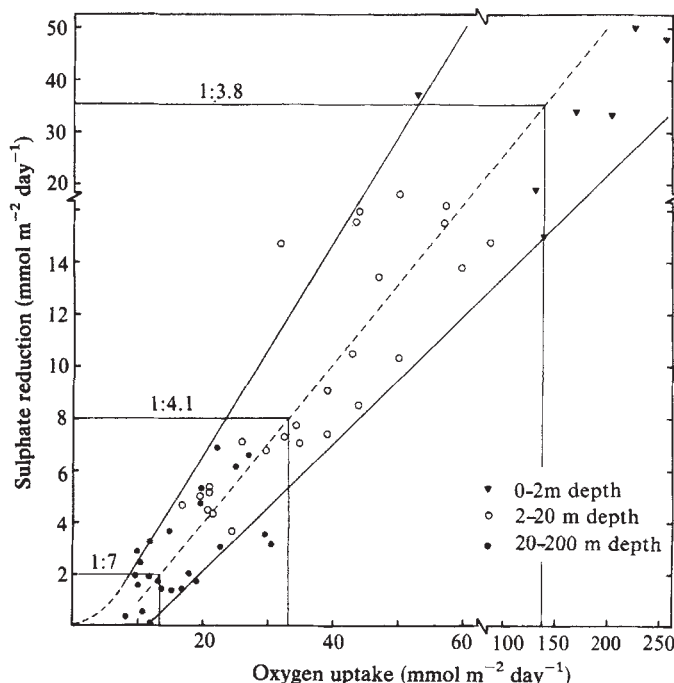


Fig. 1 Oxygen uptake and sulphate reduction rates (0–15 cm depth) of marine sediments from 0 to 200 m water depth in Kattegat, Skagerrak and Danish fjords. Sediments were carefully collected in 3–5 cm-wide coring tubes, which were kept at *in situ* temperatures under circulating, aerated water from the sampling locality and which preserved undisturbed the core stratification. Oxygen uptake was measured as the rate of disappearance of O_2 from the water over stoppered sediment cores in the dark. Experiments were stopped after 10–20% O_2 depletion to avoid decreasing uptake rates due to prolonged incubation. No bias in the $\text{O}_2/\text{SO}_4^{2-}$ ratios due to pressure release is detectable for these sampling depths. The data are averages of cores for stirred and stagnant water columns. Sulphate reduction was measured by injecting 2 μl aliquots of ^{35}S -labelled sulphate into whole cores at 1–2 cm depth intervals. Labelled sulphide was analysed after a few hours' incubation. The vertical series of individual rates were integrated with depth to give the rate per area. Each data point represents 8–50 oxygen uptake and 20–100 sulphate reduction measurements per station. Details of the experimental techniques and of potential errors are discussed elsewhere^{6,7}.

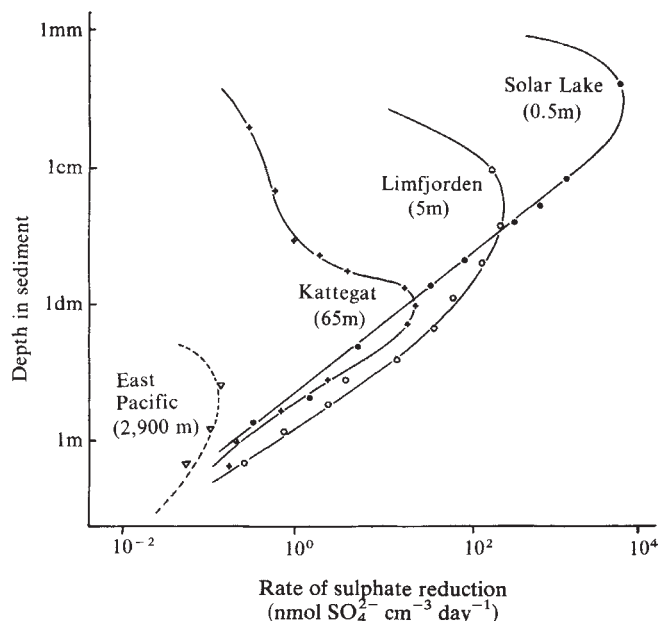


Fig. 2 Depth distribution of bacterial sulphate reduction rates for four sediment types. Water depths are indicated. Data are from Solar Lake¹⁴, Limfjorden⁶, Kattegat (B.B.J., unpublished results), and East Pacific¹⁵.

consumed by chemical or bacterial oxidation of the sulphide. The remaining 50% was used for respiration by the benthic fauna and by other aerobic organisms which use oxygen to oxidize directly the organic matter. In terms of oxidation of organic carbon, the contribution by the sulphate-reducing bacteria relative to the aerobic organisms was then ~1:1.

These conclusions generally hold for those sediments studied which have a community metabolism above 20–25 $\text{mmol O}_2 \text{ m}^{-2} \text{ day}^{-1}$ (Fig. 1). Similar calculations made for sediments at 20–200 m water depth show an average $\text{O}_2/\text{SO}_4^{2-}$ consumption ratio of 7:1 (Fig. 1). With 80% of the sulphide being re-oxidized by oxygen, ~25% of the oxygen uptake must have been consumed for H_2S oxidation. The mineralization of organic matter by sulphate reducers was thus equivalent to 35% of that directly mineralized by the aerobic organisms.

The relative importance of sulphate reduction in the shelf sediments investigated decreased with decreasing community metabolism and therefore with increasing water depth and distance from land. This may be explained by the fact that as the general metabolic rate of the sediments decreases the oxidized surface layer increases in thickness. The organic matter which reaches the deeper lying sulphide zone is therefore at a later stage of decomposition and relatively more refractory to further mineralization. The overall rate of mineralization which is expressed through the oxygen uptake consequently varies in direct proportion to the rate of organic sedimentation¹³, whereas sulphate reduction varies rather in proportion to the square of the sedimentation rate^{10,11}. Once the organic matter has reached the sulphide zone, however, it has a long residence time and can sustain the low rate of sulphate reduction over great sediment depths. The range of sulphate reduction rates calculated per unit area of the sediment is therefore much less than the range of the peak rates. Figure 2 illustrates this trend in four sediment types which differ 100,000-fold in their peak rates of sulphate reduction. In the most dynamic sediment from a small salt pond in Sinai, Solar Lake, 90% of the entire sulphate reduction occurred within the uppermost 0.1–3 cm (ref. 14). With increasing water depth, the main activity was confined to deeper but thicker sediment layers of 1–10 cm in Limfjorden⁶ and 5–20 cm in Kattegat (B.B.J., unpublished results). In sediments of the East Pacific continental slope at 2,900 m water depth, sulphate reduction was traced from a few decimetres to several metres' depth¹⁵.

Sulphate reduction in the sea bed thus occurs mainly in the coastal zone. Shelf sediments, such as those studied here from the shoreline to 200 m depth, cover an area comprising only 8.6% of the world's ocean, but $\geq 90\%$ of the oceanic sulphate reduction occurs here¹³.

The demonstrated importance of sulphate reduction in the overall mineralization of organic matter leads to a last essential point. The anaerobic bacterial food chain in which sulphate reduction is the terminal step, must be able to almost completely oxidize a wide range of organic compounds to CO₂. Newly isolated strains of sulphate-reducing bacteria can oxidize all the major fermentation products (such as C-1-18 saturated fatty acids, lactate, ethanol, succinate, and benzoate, H₂) completely to CO₂ and H₂O (ref. 16). Several of these strains have been found in high numbers in coastal marine sediments of the type studied here¹⁷. Furthermore, laboratory studies on similar sediments have shown that the lower fatty acids (acetate, propionate and butyrate) together with hydrogen³, are the main electron donors for the sulphate-reducing bacteria¹⁸. These results explain the discrepancy observed between the very restricted metabolic potential of sulphate-reducing bacteria which previously were known from marine environments (that is, *Desulfovibrio* spp.) and the evidence for an almost complete mineralization of organic matter via sulphate as demonstrated here.

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Ctenodactylid rodents in the Miocene Negev fauna of Israel

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We report here that the first rodents from the early (or early middle) Miocene of North Africa have been found in the Rotem and Yeroham Basins in the northern Negev of Israel. The rodents are associated with a large-mammal fauna resembling those of Gebel Zelten (Libya) and Moghara Wadi and Siwa Oasis (Egypt). These are the earliest records of Miocene rodents north of the Sahara, and strongly support reconstructions of a drier, more open environment in the northern part of Africa compared with the equatorial regions, with obvious significance for the evolution of grassland-adapted faunas.

As a result of two seasons (1979–80) of collecting, fossil rodents, primates and carnivores have been recovered in a new site ('Anthracothere Hill') in the northern Negev of Israel

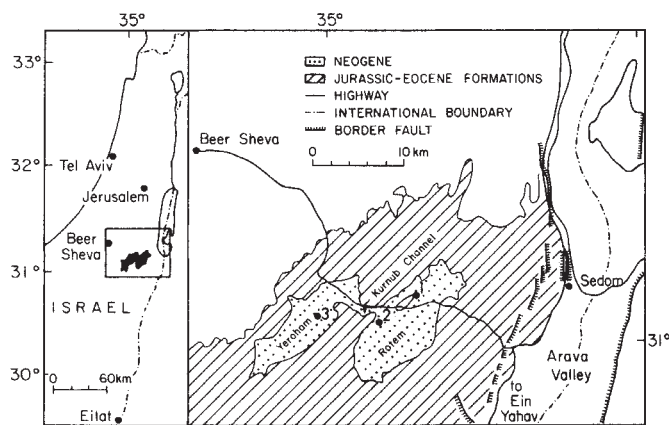


Fig. 1 Neogene basins of the northern Negev, Israel, –16–17 Myr (ref. 1).

(Fig. 1, site 1). Already these specimens have quadrupled the number of fossils known from the Negev; further study of Oron Road (site 2), which has been known for almost 30 yr, is just beginning.

Fossils of 'hippopotamus' and 'elephant' were first observed at Oron and Yeroham (site 3) during exploration for oil in the Negev during the 1950s². Savage and Tchernov³ identified these as an anthracothere and *Mastodon* (now *Gomphotherium*⁴) *angustidens* and *Deinotherium bavaricum* (now *Prodeinotherium hobleyi*⁴), and also listed rhinocerotid, tragulid and hyaenodontid specimens. Stratigraphy of sites 2 and 3 was reviewed by Neev² and Savage³, sites 1 and 2 by Hoexter and Weinberger (in preparation) and sites 1–3 by J.A.V.C. Fossils from all three sites occur in basin-filling fluvial deposits which have generally been correlated with the upper Mingar Member of the Hatseva Formation in the southern Jordan Rift Valley (Wadi Arava)⁵. We assume that deposition at these sites was approximately simultaneous, but because depositional environments are clearly different, the faunal lists are separated (Tables 1, 2).

In the Yeroham sites, specimens are found on the surface or in a poorly cemented, friable sandy matrix in low-relief badlands and gullies. An oyster reef (*Crassostrea*) 5 m above the mammalian fossils represents the maximum onlap of a marine transgression. The vertebrate fauna is depauperate, consisting of only four species (Table 1) represented by 22 specimens. Their size and shape appear to match the selection seen in Behrensmeyer-Voorhies Group III (ref. 6): a small collection of dense bones and teeth accumulated in a lag deposit.

At the Oron Road site, fossils occur just below the ground surface on the south-west-facing slope of the Rotem central arch, ~30 m south of the road. The matrix is indurated conglomerate with occasional pockets of quartzose sand. Clay balls, silt, flint cobbles and Cretaceous invertebrates from the flint occur rarely. Rare fragments of *Crassostrea* (Z. Lewy, personal communication) indicate either drainage from the Yeroham Basin, the reverse of early middle Miocene flow⁵, or the former presence of oyster reefs in eroded and redeposited parts of the Rotem sediments. Small fossils form 7% of a total of 73 specimens; no fish spines have been recovered. Large mammal bones and teeth were abraded in transport and subject to bioturbation,

Table 1 Yeroham Basin fauna

Pisces		Vertebrae
Reptilia	<i>Lates</i> sp.	
Crocodylia		
	<i>Crocodylus ?lloydi</i>	Scute, pubis, teeth
Mammalia		
	Proboscidea, indet.	Pelvis
	<i>Gomphotherium angustidens</i> *	Molar fragments, M ₁
	<i>Prodeinotherium hobleyi</i> *	Mandible, molar crowns

sp., Species unknown; indet., indeterminate.

* Ref. 3 (in part).

desiccation and fracture *in situ*. The assemblage is Behrensmeyer-Voorhies I–II–III, redeposited after bulk transport in flood conditions⁶.

At Anthracothere Hill fossils occur on the steeply inclined eastern face of a low ridge, Giv'at Kadmai. A few large fossils, for example, a rhinoceros phalange and a palm stipe, were recovered in the slope wash. Strata underlying this ridge, not fully revealed, include 7–10 m mudstone; 1–1.5 m of fossiliferous, loosely indurated red-yellow quartzose sandstone and pebbly grit in 1–2-cm layers, interbedded with massive white sandstone; and 1–8 m of coarse sand, locally crossbedded; the ground surface is Cretaceous flint cobbles. *Baculites*, *Ostrea*, *Dentalium* and *Exogyra* fragments from Cretaceous flint are rare; chalcidony cobbles to 0.5 m, calcareous pebbles, limonite nodules, clay balls to 0.2 m and silt are randomly interspersed. Sand grain analyses and upward fining of the layers indicate a high-energy palaeoenvironment, such as a river bank or channel deposit.

Small fossils, including fish bones and spines, vertebrae and teeth of small mammals, are about one-third of 445 specimens from Anthracothere Hill (Table 2). Large bones, 10–20 cm, well preserved as to detail but not robust, such as a juvenile rhinoceros mandible, a bovid tibia and several ribs, were found encased in grit. These may have been scavenged or fresh carcasses cached by crocodiles or other predators⁶. The palate of a very young post-hatchling crocodile found within one such grit layer is consistent with underwater burial. Bone pebbles 0.2–10 cm occur, but overall the large numbers and well preserved aspect of the small fossils suggest Behrensmeyer-Voorhies class I–II and deposition after brief transport.

Vertebrate microfossils in fine white sand, a palm trunk and root casts have been found in Kurnub Channel, the Miocene channel between Rotem and Yeroham Basins (Fig. 1). A humerus of the Oligocene–Miocene dugongid *Halitherium*⁷ was recently found at Ein-Yahav in the Arava. In sum, despite variations in abrasion, taphonomy and stratigraphical context, it is probable that the fossils of Yeroham, Oron and Anthracothere Hill were deposited at more or less the same

time, perhaps within a few tens of thousands of years of one another. Although direct dating of the fossils has not yet been obtained, if the strata in these basins belong to the Hatseva Formation, they are older than the faulting that now separates the basins from the type area in the Jordan Rift; the age of this rifting has been estimated from fission-track analysis of the 'Mottled Zone' at 13.6 ± 2.0 Myr (refs 8, 9). On the other hand, the marine transgression and aggradational episode to which the Yeroham oyster reef may be related, may be either that of the late early Miocene 'Vindobonian transgression' associated with the guide fossils *Orbulina* and *Borelis melo curdica* at about 15–16 Myr, which in Israel was preceded by an erosional phase and followed by a desiccation event¹⁰, or the reef-building episode in the upper Ziqim Formation of late Miocene age, ~9 Myr (ref. 11). The mammal fauna, although not highly age-diagnostic in itself, seems clearly to be of earlier rather than later Miocene age³ and similar to later early Miocene faunas of Gebel Zelten, Wadi Moghara and Siwa Oasis, as is shown below.

Two taxa of 25, *Dicerorhinus* and *Tomistoma*, are found only at Oron; four taxa of 28, bird, insectivore, primate and cricodontid rodent, are found only at Anthracothere Hill, but these differences reflect sampling intensity and taphonomy. If palaeoecology can be inferred from similar modern forms^{12,13}, the combined inventory represents five habitats: brackish lagoon (dugong, sharks); river/lake/swamp (anthracothere, crocodiles, trionychid turtle, fish, palms); forest (testudinid turtle, tragulid, proboscideans, primate, creodont, carnivore); grassland (giraffid, bovid, rhino, creodont, carnivore, cricetid and pedetid rodents); and semi-arid to rocky landscape (gazelle, ctenodactylid rodent). Continued sampling may change these econiche estimates, but the impression is a large number of habitats confined in a small terrain, and lake basins draining a few hundreds of kilometres and debouching on to a coastal corridor.

The list contains 27 African endemic taxa and what appears to be an immigrant, the ctenodactylid *Metasayimys*, represented by 12 incisors from the Rotem sites. *Metasayimys* in the Rotem

Table 2 Oron and Anthracothere Hill fauna

		Oron Road	Anthracothere Hill
Pisces			
Chondrichthyes	indet.	Teeth	Teeth
Osteichthyes	indet.		Dentary
	<i>Lates</i> sp.	Vertebra	Vertebrae, spines, preoperculum
Reptilia			
Chelonia	<i>Trionyx</i> sp.	Scutes	Scutes
	Testudinidae	Scutes	Scutes
Lacertilia	indet.	Vertebra	Vertebrae
Crocodylia	<i>Crocodylus</i> ? <i>lloydi</i>	Scutes, teeth, mandible	Scutes, teeth, mandible
	Tomistomidae	Teeth	
Aves	? <i>Passeriformes</i> sp.		Ulnar shaft
Mammalia			
Insectivora	indet.		Mandible
Primates	? <i>Cercopithecidae</i>		Teeth, bone
Rodentia	<i>Megapedetes</i> sp.	Incisors	Incisors
	<i>Metasayimys</i> sp.	Incisor	Incisors
	<i>Cricetodon</i> sp.		Incisors, mandible, molar
Creodonta	indet.	*Tooth	Mandible
Carnivora	indet.	Phalange	Phalanges
	Viverridae		Humerus
	Canidae		Humerus
Perissodactyla			
Rhinocerotidae	indet.	*Teeth, metapodial, tibia	Humerus, phalanges, mandible
	<i>Dicerorhinus</i>	Cubitus, humerus	
Artiodactyla			
Anthracotheriidae	indet.	*Teeth	
	? <i>Hyoboa</i> sp.	Premolar	Premolar
Suidae	indet.	Molar	Teeth, phalanges
Tragulidae	indet.	*Tooth	
	<i>Dorcatherium</i> sp.	Caput femoris	Teeth, calcanei
Giraffidae	indet.	Molar	P ²
Bovidae	<i>Boselaphini</i> , indet.	Tibia, calcanei, phalange	Teeth, astragalus, sacrum, tibia
	<i>Eotragus</i> sp.	Molar	Mandibles, horn, teeth, M ¹
	<i>Gazella</i> sp.	Mandible	Calcanei
Proboscidea	indet.	Vertebra	Vertebrae
	<i>Gomphotherium angustidens</i>	Molar fragments, phalange	Molar fragments
	<i>Prodeinotherium hobleii</i>	Phalange	Molar, deciduous premolar

For abbreviations see Table 1 legend.

Table 3 Negev genera: correlation with fauna from other African and European sites

	Rusinga early Miocene ~18 Myr	Gebel Zelten ?early middle Miocene ~16 Myr	Negev	Europe MN 4 ~16-17 Myr
<i>Metasayimys</i>			+	[+]
<i>Megapedetes</i>	+		+	(+)
<i>Dorcatherium</i>		+	+	+
<i>Gomphotherium</i>	+	+	+	+
<i>Prodeinotherium</i>	+	+	+	+
<i>Hyoboa</i>	+	+	+	
<i>Eotragus</i>	?	+	+	+
<i>Gazella</i>	?	+	+	
<i>Dicerorhinus</i>	+		+	

Sources: Rusinga, Kenya, ref. 23, Appendix; Gebel Zelten, Libya, ref. 28, Table 2; Negev, Israel, this work; Europe, refs 14, 26 Table; [] ctenodactylid sp., Pasalar, Turkey, ref. 15; () Bayraktepe, Turkey, ref. 24, possibly younger, see text.

sediments may be the earliest African appearance, if this fauna proves to correlate with MN zone 4 (ref. 14, see below). The next youngest record of this group is 'Ctenodactylid, sp. indet.' at Pasalar, Turkey¹⁵, in zone MN 5 and the equivalent, or slightly younger, *Metasayimys cf. intermedius* of the Dam Fm, As Sarrar, Saudi Arabia (H. Thomas, personal communication). Related species are *Metasayimys intermedius* Sen and Thomas¹⁶ of the Hofuf Fm, Hasa Province, Arabia, "older than ... Beni Mellal (our translation)"; *Metasayimys jebeli Lavocat*¹⁷ of Beni Mellal, Morocco, and *Sayimys* from the Chinji Fm, lower Siwaliks, India^{18,19}, also about this age.

Hyoboa and *Dicerorhinus* have been considered slightly earlier immigrants from Asia, observed at Dera Bugti, Pakistan²⁰⁻²², but as both appear at the approximately contemporaneous early Miocene sites in Kenya²³, an African origin seems equally probable. The creodonts and carnivores of Rotem-Yeroham (in preparation) also have African early Miocene affinities. African emigrants into Eurasia in the early Miocene are *Dorcatherium*, *Gomphotherium*, *Prodeinotherium* and *Eotragus*. *Megapedetes*, also emigrant, has been recovered at Bayraktepe, the southern Dardanelles, Turkey²⁴, with a mix of faunal elements, some as young as ~13 Myr (for example, *Hispanomys*).

To determine biogeographic correlation of the Negev fauna, we relied on radiometric- and marine-controlled dating of Rusinga²⁵, Europe^{14,26}, the Mediterranean²⁷ and a transect of the Israel shoreline¹⁰. The major datum points are the Rusinga lavas at ~18 Myr and the appearance of the foraminifer *Orbulina suturalis* at ~16 Myr; Gebel Zelten is set at ~16 Myr.

In Table 3, based on small-to-large herbivores, we equate the Negev basins and Gebel Zelten, Libya, and by extension, Moghara and Siwa, Egypt²⁸⁻³⁰. (Determination of the Rotem-Yeroham assemblage seems to be slightly older than that of As Sarrar in western Arabia (H. Thomas, personal communication) and ranked by age, older than the fauna of Al Jadidah and Ab Dabtiyah, Saudi Arabia, the India-Pakistan sites, and Fort Ternan, Kenya, the last dated 14 Myr (refs 31-35). *Hyoboa* and *Dicerorhinus* suggest that Rotem-Yeroham is younger than Dera Bugti^{21,22} and Rusinga²³.

The best estimate for the Negev fauna is thus ~16-17 Myr, a time of global warming and land emergence observed in the Mediterranean Sea and at continental margins^{10,36,37}. The thermal high that began in the earliest Miocene was just at peak and Israel shore emergence was maximal. Cogley's reconstruction of the eastern proto-Mediterranean ~20 Myr (J. G. Cogley, personal communication) shows a filling-in between Israel and Turkey which agrees with the ctenodactylid appearance at Pasalar. Except for the faunal comparisons, however, the early middle Miocene has revealed no compelling geological evidence for a connection between Negev basins and Europe via Turkey.

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Free Ca²⁺ and cytoplasmic streaming in the alga *Chara*

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Plant cells, like those of animals, contain the Ca²⁺-binding protein calmodulin¹⁻⁴. By analogy with animal cells it has thus been suggested that the intracellular free Ca²⁺ concentration may have an important role in the regulation of plant cell activities. This suggestion has been supported by various physiological experiments, but so far direct evidence, involving measurements of intracellular Ca²⁺ levels, has not been obtained. We describe here measurements of intracellular Ca²⁺ in the giant alga *Chara* by microinjection of the protein aequorin, which emits blue light in proportion to Ca²⁺ concentration. *Chara* exhibit an ATP-dependent cytoplasmic streaming shown to be inhibited by Ca²⁺ (refs 5, 6). We report that *Chara* cells have a low free Ca²⁺ concentration, comparable with those of animal cells, and that action potentials which inhibit cytoplasmic streaming⁷ increase this Ca²⁺ concentration substantially.

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Aequorin was injected into internodal cells of *Chara corallina* or *Nitella* sp., and initially showed a high light emission that, except for occasional transient increases, declined rapidly for 30–50 min (Fig. 1a). This probably reflected a relatively high cytoplasmic free Ca^{2+} concentration ($\text{Ca}_{\text{cyt}}^{2+}$) as a result of the injection process, and precluded any detailed interpretation. In the next phase the light signal was low, subsequently declined very slowly and showed no transient increases in an undisturbed, undamaged cell (Fig. 1a). This is consistent with the injected aequorin now being evenly distributed by cytoplasmic streaming and with $\text{Ca}_{\text{cyt}}^{2+}$ having declined to a low value, and this conclusion is supported by the finding that cells lysed in a solution containing a Ca^{2+} saturating for aequorin (solid line, Fig. 1b) contained active aequorin for up to 14.5 h after injection.

The peak lysis signal after correction for poor mixing (L_{max} in saturating Ca^{2+}) and the *in vivo* aequorin signal (L in $\text{Ca}_{\text{cyt}}^{2+}$) were used to determine $\text{Ca}_{\text{cyt}}^{2+}$ from a calibration curve relating L/L_{max} to free Ca^{2+} *in vitro* (Fig. 1b,c). The solutions used for this curve resembled characean cytoplasm in pH and free Mg^{2+} (Fig. 1c). Here two values for free Mg^{2+} were used because of the range reported for ATP, probably the major Mg^{2+} chelator. From the 3 mM free Mg^{2+} curve, the mean $\text{Ca}_{\text{cyt}}^{2+}$ was found to be 0.22 μM for *Chara* (range 0.1–0.56 μM , $n=6$) and 1.1 μM for *Nitella* (range 0.79–1.6 μM , $n=4$). At 1 mM free Mg^{2+} , the mean values were lower, that is, 0.1 μM and 0.44 μM , respectively. Cells appeared undamaged by several physiological criteria and neither chloroplast light absorption nor partial aequorin inactivation by enzyme inhibitors generated at cell lysis introduced major inaccuracies into the estimates of $\text{Ca}_{\text{cyt}}^{2+}$. In addition, these values agree with those suggested by cell perfusion experiments^{5,6,8,9}.

Uncentrifuged cells were also injected with aequorin using a large tipped micropipette which penetrated the vacuole through $\sim 10 \mu\text{m}$ of parietal cytoplasm¹⁰. The resulting relatively rapid light emission left little or no unreacted aequorin detectable on cell lysis after 15 min while unreacted aequorin was present 14.5 h after injection into the cytoplasmic compartment. The half time for aequorin utilization in a medium of sap expelled from cut cells indicated a near saturating Ca^{2+} ($L/L_{\text{max}}=0.56$). Any vacuolar inhibitors, including low vacuolar pH (Fig. 1 legend and ref. 11) will make this an underestimate of vacuolar Ca^{2+} , suggesting that the free Ca^{2+} is $>10^{-4} \text{ M}$ in this compartment.

Thus characean cells contain a cytoplasmic compartment where free Ca^{2+} is maintained at a concentration of micromolar or less and a vacuolar compartment where the free Ca^{2+} is $>10^{-4} \text{ M}$. These measurements of free Ca^{2+} should be compared with measurements of total Ca^{2+} for characean cells indicating 3–12 mM in the vacuole^{12–15} and 2–8 mM in the cytoplasm^{6,15}.

On electrical or mechanical stimulation, characean cells propagate action potentials along both the plasma and tonoplast membranes¹⁰ and this is associated with streaming cessation⁸. The ability of elevated Ca^{2+} concentration to inhibit both streaming in perfused characean cells^{5,6,16}, as well as chloroplast rotation in cytoplasmic fragments¹⁶ isolated from characean cells, makes a rise in $\text{Ca}_{\text{cyt}}^{2+}$ a likely explanation for streaming cessation by the action potential in intact cells⁸.

Chara cells with cytoplasmic aequorin were stimulated with external electrodes and changes in vacuolar potential (Ψ_{vo}) and either aequorin light emission or streaming velocity were measured. The electrical transient was accompanied by a large

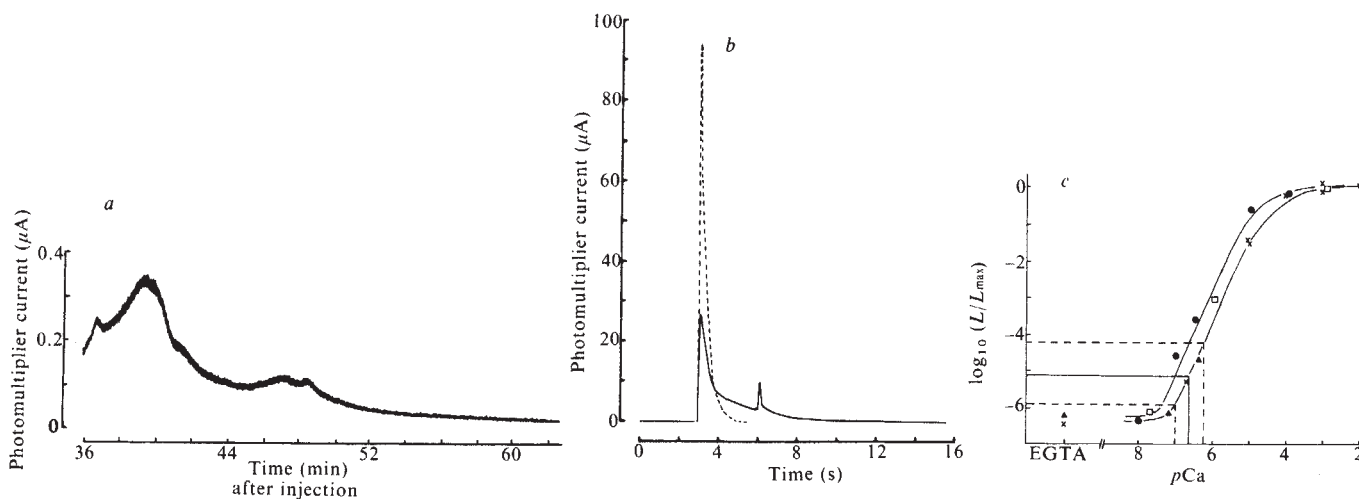


Fig. 1 Aequorin prepared as in ref. 35 was dissolved in (mM): 150 KCl, 10 *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid (Na_2TES), pH 7.5, and $\sim 50 \text{ nl}$ (10% cytoplasmic vol) was injected into an internodal cell ($\sim 40 \text{ mm}$ long) of *Chara corallina* or *Nitella* sp. which had been centrifuged at low speed for several minutes, and immersed, to reduce turgor, in (mM): 0.1 KCl, 1.0 NaCl, 200 or 250 sucrose, with the centrifugal end exposed. Aequorin was injected into the resulting endoplasmic plug, the pipette withdrawn, and the cell returned to full turgor by diluting out sucrose with artificial pond water (APW) (mM): 0.1 KCl, 1.0 NaCl, 0.1 CaCl_2 , 2 Na_2TES , pH 7.2). Aequorin light emission was detected with an EMI 9635A (-900 or -1400 V) photomultiplier (signal output to a chart recorder via current-voltage converter ($\text{RC} = 200 \text{ ms}$ or 0.1 ms) protected from ambient light by a shutter; (bar on time axes denotes shutter closure). All photomultiplier currents were standardized by conversion to their equivalent current at $-1,400 \text{ V}$ with photomultiplier dark current (4.5 nA) and chamber ambient light (shutter open, uninjected cell, $\sim 0.5 \text{ nA}$) subtracted. **a**, Aequorin light emission from cytoplasmically injected *Chara* cell. **b**, Light emission (solid line) following crushing of similar cell in a lysis solution (mM): 10 CaCl_2 , 50 Na_2TES , 1% (v/v) Triton X-100, pH 7.3. Rapid, complete aequorin discharge required both crushing and detergent. L_{max} for $\text{Ca}_{\text{cyt}}^{2+}$ calculation (ref. 45 and fig. 1c) requires correction for poor mixing as in the broken curve enclosing same area and with $t_{1/2} = 0.49 \text{ s}$ ($t_{1/2}$ measured *in vitro* when aequorin is rapidly mixed with lysis solution at $\sim 20^\circ\text{C}$ (see ref. 35)). **c**, *In vitro* calibration curves for measuring $\text{Ca}_{\text{cyt}}^{2+}$ from L/L_{max} . 50 nl of aequorin solution was quickly mixed (see ref. 35) with either lysis solution (saturating, 10^{-2} M CaCl_2) or with solutions containing (mM): 120 KCl, 1 (●, □) or 3 (×, ▲) free Mg^{2+} , 30 Na_2TES , pH 7.6 and various Ca^{2+} concentrations achieved below 10^{-4} with EGTA. Mg^{2+} concentration approximated to cytoplasmic free Mg^{2+} calculated as total free Mg^{2+} (3.6 mM; ref. 5) minus free ATP (within range $0.5\text{--}1.0 \text{ mM}$ ^{46,47} but values up to 4.0 mM have been reported⁴⁸). The pH is within the range reported using weak acid and microelectrode methods⁴⁹. Fractional luminescence (L/L_{max} ; ref. 45) is the ratio of peak light intensity in non-saturating (L) and saturating (L_{max}) Ca^{2+} concentrations, plotted as a function of free Ca^{2+} . $\text{Ca}_{\text{cyt}}^{2+}$ for injected cells was calculated from L/L_{max} where L was the signal from cell before lysis and L_{max} the peak signal on lysis in a saturating free Ca^{2+} after correction for slow mixing (Fig. 1b, dotted line). (T , 20°C). Possible sources of error are the following. (1) Injection damage of cell elevating the $\text{Ca}_{\text{cyt}}^{2+}$; injected *Chara* cells propagated action potentials (Fig. 2a) and showed normal membrane potentials and streaming velocity (107% and 96% respectively of uninjected control values). (2) Chloroplast light absorption may differ in intact and lysed cells, distorting L/L_{max} : light from an aequorin- Ca^{2+} sample in a micropipette was reduced about fivefold on insertion in a *Chara* cell with undamaged chloroplast array (see ref. 5). However, as $L \propto [\text{Ca}^{2+}]^2$ (ref. 45 and Fig. 1c) (given L fully shielded and L_{max} unshielded), true $\text{Ca}_{\text{cyt}}^{2+}$ would be 0.44 times the calculated value. (3) Subcellular compartmentation breaking down at lysis may generate enzyme inhibitors and reduce L_{max} . In *Nitella*, aequorin mixed with sap from cut cells gave 82% of light recorded from aequorin with lysis solution. Inhibition was mainly due to sap acidity as judged by repeating observation in presence of sufficient TES buffer to maintain pH 7.2. With $L \propto [\text{Ca}^{2+}]^2$, true $\text{Ca}_{\text{cyt}}^{2+}$ is $0.92 \times$ calculated $\text{Ca}_{\text{cyt}}^{2+}$ in worst case where aequorin was discharged at cell lysis by non-pH buffered vacuolar sap.

increase in light emission (Fig. 2a) which was not seen if uninjected cells were used. As in uninjected cells, the detailed shape of the electrical record varied from cell to cell, and to some extent with time in an individual cell, and the light emission varied similarly. Action potentials, which were all-or-none and typical of the characeae, could be elicited repeatedly over several hours with only a gradual decline in the amplitude of the aequorin signal resulting from an estimated 4% of the aequorin being consumed by each action potential response (Fig. 2 legend). The peak light emission corresponds closely to the visually estimated time for streaming cessation (Fig. 2a; see also ref. 17). However, aequorin light returns to its resting level in 40–60 s, while streaming in different cells may take 75–360 s and have only reached some 25% of its full rate by the time aequorin light has fully recovered (Fig. 2b). If aequorin light accurately reflects changes in $\text{Ca}_{\text{cyt}}^{2+}$, then a hypothesis of Ca^{2+} control requires a mechanism in which there is rapid inhibition by elevated $\text{Ca}_{\text{cyt}}^{2+}$ but slow recovery extending many seconds beyond the recovery time for $\text{Ca}_{\text{cyt}}^{2+}$. In vertebrate smooth muscle, where the time course of $\text{Ca}_{\text{cyt}}^{2+}$ and tension also diverge¹⁸, free Ca^{2+} is thought to exert control via a calmodulin-dependent myosin light-chain kinase, causing phosphorylation which, in turn, is regulated by a cyclic AMP-dependent kinase followed eventually by a slow dephosphorylation step^{19–24}. The existence of a similarly complex regulatory system in *Chara* may generate the substantial divergence observed between the recovery of streaming and of aequorin light.

We can also determine the magnitude of the free Ca^{2+} change as a result of the action potential. $\text{Ca}_{\text{cyt}}^{2+}$ was calculated to rise 30-fold to 6.7 μM (mean of 6 cells) during a *Chara* action potential (from Fig. 1c taking $L\propto[\text{Ca}]^2$) (42-fold for *Nitella* to 43 μM , $n=4$). This would take $\text{Ca}_{\text{cyt}}^{2+}$ into the range where ATP-dependent streaming in perfused cells is strongly inhibited by Ca^{2+} (refs 5, 6, 25) but it is still less than the 10^{-3} M Ca^{2+} needed to stop streaming completely in perfused cells⁸, as happens at the action potential¹⁷. This discrepancy implies (1) that there is another inhibitor of streaming associated with the action potential (perhaps, a change in cytoplasmic pH); (2) that the cell model is depleted by perfusion of some as yet uncharacterized regulatory protein, and is thus less sensitive to Ca^{2+} than the intact cell; or (3) that the peak $\text{Ca}_{\text{cyt}}^{2+}$ has been underestimated. This would occur if aequorin light emission were inhibited by, for example, a rise in the concentration of H^+ or free Mg^{2+} , if $\text{Ca}_{\text{cyt}}^{2+}$ did not increase in all parts of the cytoplasm, or if it increased asynchronously in different regions (see, however, Fig. 2 legend). The return of aequorin light to precisely its pre-stimulus value (Fig. 2b) indicates, however, that if any ionic changes affecting the sensitivity of aequorin occur, they must be short-lived.

Replacing external Ca^{2+} with Mg^{2+} leads to a reversible loss of excitability in *Chara* which may be due to a specific requirement for Ca^{2+} to initiate the transient change in anion permeability²⁶. Mg^{2+} treatment of cells containing cytoplasmic aequorin led to partial depolarization and to a reversible decline in the magnitude of both the electrical and aequorin-light transients (Fig. 3a). This was accompanied by a loss of streaming inhibition which returned with the electrical and aequorin transients when Ca^{2+} was restored to the bathing solution. There appeared to be a degree of separation between changes in the electrical transient and the changes in the aequorin light response (Fig. 3a), with the alterations in electrical responsiveness preceding the changes in aequorin light emission during both the loss and recovery phases. The partial recovery of the electrical transient before the light transient is consistent with the idea that other ions^{14,26,27} besides Ca^{2+} have a role in the electrical transient. In *Nitella*, complete replacement of Ca^{2+} by Mg^{2+} results in no loss of excitability, a prolongation of the electrical response and no loss of streaming²⁸, suggesting that cytoplasmic Ca^{2+} stores are less likely to be a source of the changes in $\text{Ca}_{\text{cyt}}^{2+}$; the results in Fig. 3a also support this notion, as do tracer influx experiments⁸.

One batch of *Nitella* did not show the typical characean

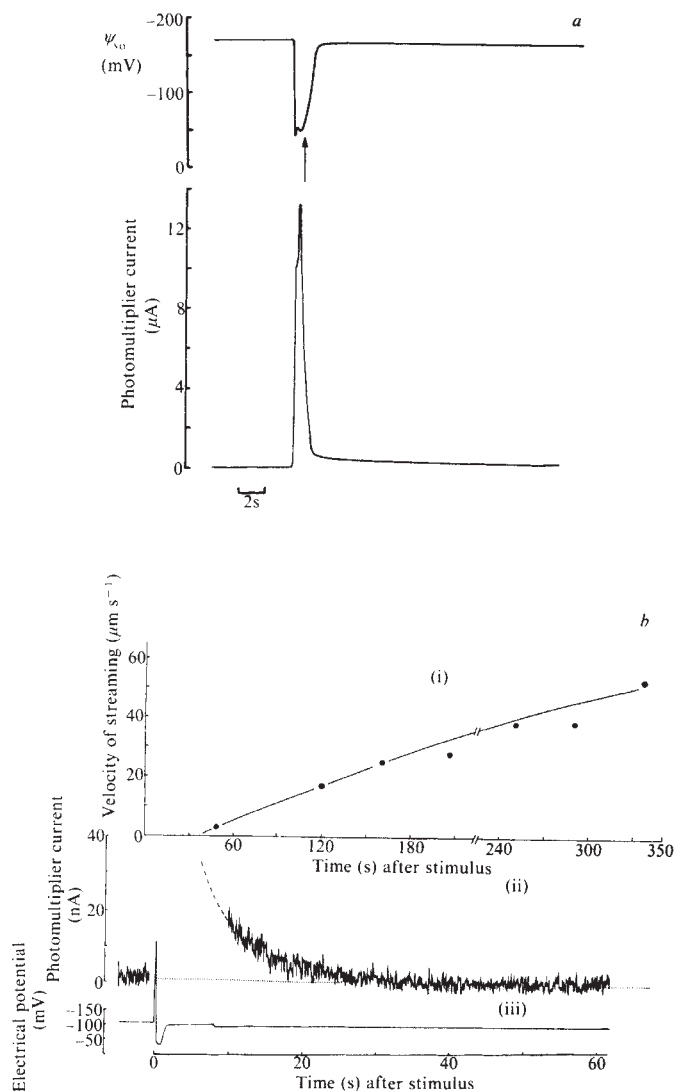


Fig. 2 a, Changes in electrical potential (Ψ_{vo}) and aequorin light emission during an action potential. The 'tail' to the electrical record indicates that the electrode tip is in the vacuole¹⁰. Current pulses from a Devices Stimulator were applied to cell via full length external Ag/AgCl wire electrodes immersed in pools of APW, separated by a silicone grease barrier. The microelectrode signal was fed to a Keithley electrometer and then to a Tekman TE220 chart recorder. Either aequorin light emission, recorded from 90% of the cell length by a standard photomultiplier arrangement, or cytoplasmic streaming measured close to the microelectrode, were determined. Streaming cessation (determined visually) occurred ~300 ms after maximum depolarization (arrow). Calculations indicated that 3.7% ($n=4$) of the injected aequorin is consumed during each action potential. b, Recovery of aequorin light emission (ii) to pre-stimulus level following an action potential indicated on the record of vacuolar potential (Ψ_{vo}) (iii). (Output from photomultiplier was shorted out during the stimulus to show recording at high gain during the recovery phase). The photomultiplier current includes resting light from injected cell (~2 nA) (see Fig. 1 legend). (i) Indicates that the recovery of streaming velocity to its pre-stimulus value of ~50 $\mu\text{m s}^{-1}$ was much slower than recovery of the aequorin light emission. Observations are from the same cell. T, 20 °C. The cessation of chloroplast rotation at the action potential⁸ indicated that motility inhibition occurred at all depths in the endoplasm. However, the inhibition spreads radially inwards through the endoplasm at 15 $\mu\text{m s}^{-1}$ so that, if the increase of $\text{Ca}_{\text{cyt}}^{2+}$ is being observed, all regions will be affected but not synchronously. Radial spread through a typical thickness of endoplasm would take ~0.5 s (ref. 10). However, the time between the stimulus artefact and the membrane response recorded at the further end of the cell suggest that the cell was excited almost simultaneously by the extensive external electrodes. While some asynchrony and therefore underestimation of the peak $\text{Ca}_{\text{cyt}}^{2+}$ seems possible, the consumption of only 4% of the total aequorin during each action potential severely restricts the time for which the 10^{-3} M $\text{Ca}_{\text{cyt}}^{2+}$ required for complete inhibition in perfused cells could exist. As $t_{1/2}$ for aequorin reaction is ~0.5 s at 10^{-3} M Ca^{2+} , 4% of the aequorin would be consumed in 40 ms if this concentration was reached instantaneously. If allowance is also made for aequorin consumption during the finite rising and recovery periods, the existence of 10^{-3} M Ca^{2+} would have to be even briefer.

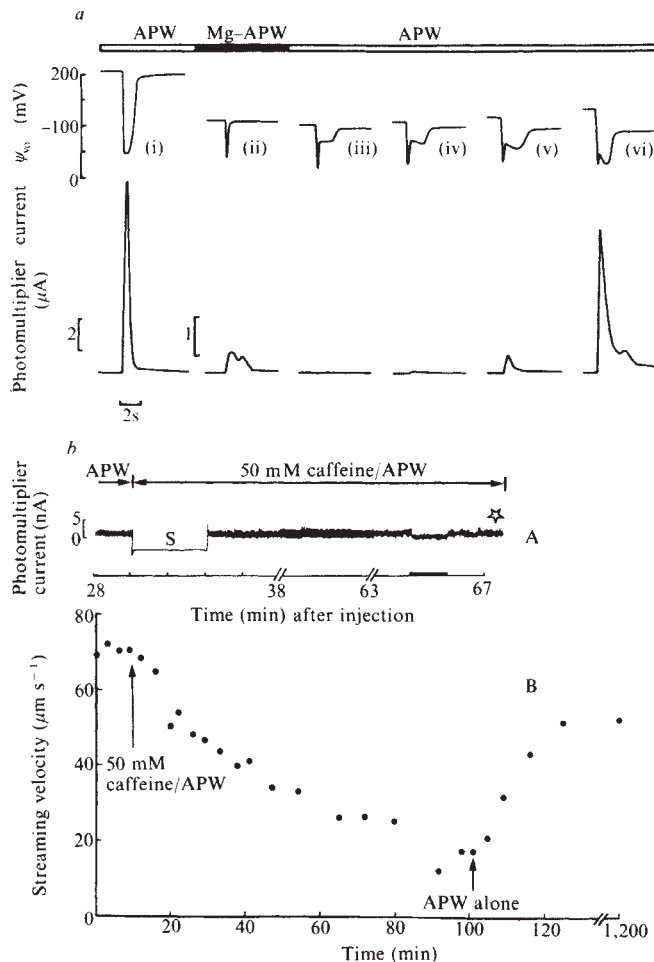


Fig. 3 *a*, Traces of vacuolar electrical potential (ψ_v) and aequorin light from *Chara* cell exposed for 26 min to Mg-APW (mM): 10 MgCl₂, 1 NaCl, 0.2 KCl, 2 Na₂TES, pH 7.2. Note that the electrical transient is preceded by a stimulus artefact clearly seen in trace (ii) when the transient has been abolished. (ii) Mg-APW led to loss of electrical response and decline in aequorin light response that was complete by the time APW was restored (trace iii). Recovery of the electrical and aequorin light response (traces iii-vi) documented 13, 15, 24 and 40 min after return of APW showed recovery of the aequorin light response to be slower than the electrical response as substantial electrical transients were observed in APW before a significant recovery of aequorin light response. Inhibition of streaming on stimulation was lost in Mg-APW, returning only slowly in APW. After 24 min APW (trace v) there was no inhibition 30 s after stimulation; after 50 min in APW, streaming inhibition was complete (trace very similar to trace vi) but recovery was more rapid than normal (~75 s, see Fig. 2*b*). *b*, Inhibition of cytoplasmic streaming (B) in *Nitella* by 50 mM caffeine-APW was accompanied by no appreciable increase in aequorin light emission (A). The streaming record is from an uninjected cell but examination of the injected cell at the end of the light emission trace (☆) revealed inhibited streaming which recovered on replacing the caffeine with APW. The artefact (S) on trace A represents a solution change with the photomultiplier output shorted out.

all-or-none behaviour¹⁰. Here stimuli of increasing magnitude evoked aequorin light transients of increasing size and resulted in correspondingly more marked inhibitions of cytoplasmic streaming^{29,30}. These results suggest that cytoplasmic streaming responds to Ca^{2+}_{cyt} levels intermediate between those found in the resting cell and those achieved at the normal action potential, behaviour which is qualitatively consistent with the response of perfused cells to such variations in free Ca^{2+} (refs 5, 6, 25).

Finally, *Nitella* cells were exposed to caffeine, which in muscle is able to release Ca^{2+} from the sarcoplasmic reticulum and cause contraction³¹. In plants, caffeine inhibits cytokinesis³², vesicle secretion³³ and chloroplast rotation³⁴. Caffeine inhibited cytoplasmic streaming in *Nitella* (Fig. 3*b*), although only at the rather high concentrations needed to stop chloroplast rotation in *Coleochaete*³⁴. However, the inhibition of streaming in *Nitella*

was not accompanied by any substantial increase in aequorin light emission (Fig. 3*b*). Thus caffeine inhibits a Ca^{2+} -sensitive plant process, but apparently not by appreciably increasing Ca^{2+}_{cyt} .

Ca^{2+}_{cyt} in the two characean algae studied is some 10^4 times lower than total cytoplasmic Ca^{2+} . While clearly an extrapolation, it seems likely that all plant cells will conform to this low Ca^{2+}_{cyt} pattern as have the diverse animal cells studied to date³⁵.

Given these low Ca^{2+}_{cyt} values, the external 10^{-4} M Ca^{2+} and 10^{-4} M Ca^{2+} in the vacuole provide large electrochemical gradients for Ca^{2+} entry into the cytoplasm at both the plasma membrane (cytoplasm ~170 mV negative) and the tonoplast (cytoplasm ~20 mV negative¹⁰). Although the measured Ca^{2+} influx at the plasma membrane is small (~ 0.04 pmol $cm^{-2} s^{-1}$)^{8,15}, in isolation this would raise Ca^{2+}_{cyt} at the rate of $0.04 \mu M s^{-1}$, a large change in relation to the resting Ca^{2+}_{cyt} ; this calculation assumes a $10 \mu m$ -thick layer of cytoplasm and no binding or sequestration. Our results indicate that sequestering by cytoplasmic organelles may provide short-term control of Ca^{2+}_{cyt} . Long-term stability would require Ca^{2+} movement either directly out of the cytoplasm across the plasma membrane or into the large vacuolar reservoir, presumably ultimately effluxing to the external medium; a flux of 0.04 pmol $cm^{-2} s^{-1}$ would increase the total Ca^{2+} by ~ 1 mM in 7 h. In a situation where several organelles plus the plasma membrane and tonoplast may contribute to maintaining Ca^{2+}_{cyt} , the quantitative importance of each component process *in vivo* is at present difficult to predict.

The range of Ca^{2+}_{cyt} changes reported here corresponds well to the operating range for calmodulin and other Ca^{2+} -regulated proteins⁵⁰ and suggests that changes in Ca^{2+}_{cyt} could regulate, either directly or indirectly, many aspects of plant cell biology. So far the action potential is the only proven effector of a change in plant Ca^{2+}_{cyt} and it remains to be determined whether action potentials in other plant cells^{36,37} use Ca^{2+}_{cyt} to exert their postulated effects on processes such as morphogenesis^{38,39}. Similarly, cytoplasmic streaming is the only physiological process known to be affected by that Ca^{2+}_{cyt} change, although other Ca^{2+} effects have been postulated for characean cells, such as the possibility that light and plant hormones are also able to change Ca^{2+}_{cyt} (Refs 40-44). It is now possible with this method to investigate these suggestions more precisely.

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Non-coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxins

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The binding of various biologically significant macromolecules to specific cell surface receptors is followed by their internalization, a process called receptor-mediated endocytosis^{1,2}. In most cases, it has been shown that receptor-bound ligands cluster in characteristic, bristle-coated indentations of the cell surface known as coated pits^{1–3}. In addition to coated pits, cultured cells have a population of smaller, non-coated membrane invaginations^{4–6}, which may have a role in endocytosis^{2,7}. However, no receptor-bound biologically active ligand has been shown to enter cells via these non-coated invaginations. The studies implicating coated pits in receptor-mediated endocytosis concern essentially ligands that bind to receptors thought to be glycoproteins. We have therefore investigated by electron microscopy the endocytosis of cholera toxin and tetanus toxin, ligands which bind to membrane glycolipids, in particular to either GM₁ monosialoganglioside^{8–11} or di- and trisialogangliosides^{12–15} respectively. We show here that in cultured liver cells, non-coated membrane microinvaginations are preferentially involved in both the initial binding and subsequent internalization of colloidal gold-labelled cholera and tetanus toxin.

The rat liver cell line KLTRYPV¹⁶ (provided by Dr G. Vergani) was maintained in monolayer culture as described elsewhere¹⁷. The cells were incubated with gold-labelled toxins as described in Fig. 1 legend, then washed three times with ice-cold phosphate-buffered saline (PBS) and either immediately fixed at 2 °C in 2% glutaraldehyde in PBS for 15 min, or warmed to 37 °C for 10 or 30 min before fixation. The cultures were further processed *in situ* for electron microscopy, as described previously¹⁸.

The distribution of cholera toxin–gold and tetanus toxin–gold complexes at 2 °C on thin sections cut perpendicularly to the plane of the monolayer was essentially similar. Gold particles were scattered at irregular intervals along the plasma membrane, including the microvilli, but were frequently associated with non-coated, flask-shaped microinvaginations (Fig. 1*a, b*). In contrast, coated pits were labelled only very rarely. The association of toxin–gold complexes with the small, non-coated invaginations was also apparent in sections parallel to the substratum, in regions where the membrane had been cut tangentially (Fig. 1*c, d*). To check the specificity of labelling, cells

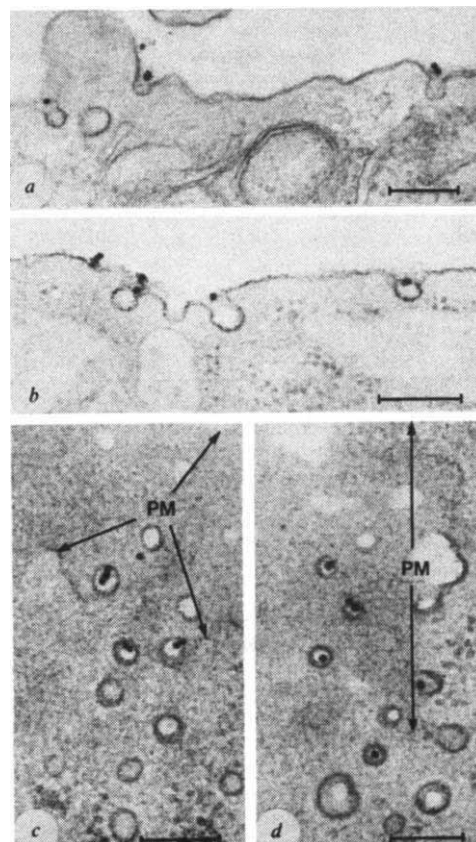


Fig. 1 Labelling pattern in cultured liver cells exposed to cholera toxin–gold (*a, c*) or tetanus toxin–gold (*b, d*) complexes at 2 °C. The preferential labelling of non-coated, flask-shaped microinvaginations is evident in sections cut either perpendicularly (*a, b*) or tangentially (*c, d*) to the plasma membrane (PM). As seen in *a* and *b*, gold particles are mostly located at the neck of the microinvaginations in these conditions. Colloidal gold (particle diameter ~15 nm) was prepared according to Frens²⁸ and the pH adjusted to 6.9 with 0.2 M K₂CO₃. Cholera toxin (Schwarz/Mann, New York) and tetanus toxin (given by Dr B. Bizzinni) were brought to 5 mM NaCl by passage through a Sephadex G-25 column (0.9 × 10 cm) equilibrated and eluted with 5 mM NaCl. The minimum amount of cholera toxin or tetanus toxin needed to stabilize a given volume of colloidal gold was estimated according to Roth and Binder²⁹. The crude toxin–colloidal gold preparations were centrifuged at 60,000 g at 4 °C for 1 h and resuspended in phosphate-buffered (50 mM, pH 7.4) isotonic saline to a final concentration of 100 µg cholera toxin per ml or 1 mg tetanus toxin per ml. Cells grown to confluency in Linbro multi-well plates (Flow) were washed three times with ice-cold PBS and equilibrated at 2 °C for 5 min before incubation with cholera toxin–gold or tetanus toxin–gold at 2 °C for 30 min. Scale bar, 0.2 µm.

were first incubated at 2 °C for 30 min with either unlabelled cholera toxin (500 µg ml⁻¹) or tetanus toxin (2.5 mg ml⁻¹) in PBS, then exposed to the toxin–gold complexes. In such conditions, surface labelling was virtually absent.

In liver cells warmed to 37 °C for 10 or 30 min before fixation, gold particles were found in small non-coated cytoplasmic vesicles (Fig. 2*a*), in larger vacuoles having the morphology of multivesicular bodies (Fig. 2*b*) and in typical lysosomes. Coated pits or vesicles were only rarely labelled. To ensure that coated pits were functional in these cells, experiments similar to those described above were done after indirect labelling of concanavalin A (Con A) binding sites with Con A followed by horseradish peroxidase (HRP)–gold¹⁹. Labelling of coated pits and vesicles was commonly observed with this ligand (data not shown).

The distribution of cholera and tetanus toxin surface labelling was quantified by calculating the ratio between the percentage of gold particles bound to each membrane specialization and

Table 1 Association of cholera toxin-coated gold particles with cell surface features

Cell surface feature	% Gold particles associated with the feature	% Surface length occupied by the feature	Ratio
Flat plasma membrane	34.1	55.5	0.6
Microvilli	30.1	34.5	0.9
Smooth	35.8	8.9	4.0
microinvaginations			
Coated pits	0	1.1	0

The numbers of gold particles found on flat regions of plasma membrane, microvilli, smooth microinvaginations and coated pits on the apical cell surface were counted directly in the electron microscope on sections cut perpendicularly to the substratum. In each case, 35–50 cells (~1.0–1.7 mm of cell surface) were evaluated. Each cell was then photographed and the length of the whole apical cell surface and the length of each surface feature were measured on negatives projected at an appropriate magnification ($\times 46,000$ for flat membrane and microvilli; $\times 92,000$ for smooth and coated invaginations) on a graphic tablet connected to an IMSAI microprocessor. The relationship of cholera toxin-coated gold particles to the various features of the cell surface at 2 °C is expressed as the ratio between the percentage of particles associated with a given feature and the percentage of the length of the cell surface occupied by the feature. A ratio of ~1 means that labelling is random. A ratio <1 suggests an exclusion; a ratio >1 indicates a preferential association.

the per cent length of cell surface occupied by that structure. The only preferential binding (with a ratio of 4) of gold-labelled cholera toxin at 2 °C was to the non-coated small membrane invaginations (Table 1). For tetanus toxin, we found a similar but less pronounced initial preferential binding (ratio of 1.5) to non-coated membrane invaginations (see Fig. 3). Furthermore, our qualitative observation that in cells incubated at 2 °C the neck of the flask-shaped membrane invaginations was more frequently labelled than the body region (compare Fig. 1a, b) was confirmed quantitatively (Fig. 3). In view of the rigidity of the membrane at 2 °C, this phenomenon could result from an impaired transfer of the gold tracer into the body of the invaginations. In contrast, after 10 min, and more markedly after 30 min of warming to 37 °C, gold particles were preferentially associated with the body of the smooth microinvaginations; the association of the label with microinvaginations as a whole was also more pronounced than at 2 °C. Cholera toxin surface labelling was reduced by only 1% after 10 min of reincubation to 37 °C, and by 26% after 30 min, indicating a slow rate of internalization. The decrease of surface labelling at 37 °C was more rapid in the case of tetanus toxin (34% after 10 min, 77% after 30 min).

In conclusion, our results show that: (1) at 2 °C toxin-gold complexes bind preferentially to non-coated, flask-shaped microinvaginations; (2) this preferential association becomes more pronounced after warming of the cell to 37 °C and is accompanied by a progressive translocation of the label from the neck to the body of the invaginations; and (3) at 37 °C the label is internalized via small, non-coated vesicles, probably derived from the surface microinvaginations, and accumulates in multivesicular bodies and lysosomes. We cannot, however, exclude the possibility that a small fraction of the label may enter cells through coated pits in the first few minutes after warming to 37 °C, as we did not study time points earlier than 10 min.

Studies on a variety of systems have contributed to the now widely accepted view that receptor-mediated endocytosis proceeds through clathrin-coated pits on the cell surface^{1–3}. These studies concern essentially ligands that bind to membrane receptors thought to be glycoproteins^{1–3}. However glycolipids, and in particular gangliosides, can also serve as selective receptor sites for various macromolecules^{20–22}, including cholera^{8–11}

and tetanus^{12–15} toxins, but the surface events involved in the internalization of these ligands have received only limited attention^{23–25}. Joseph *et al.*²⁴ mentioned that cytoplasmic coated vesicles did not participate in the internalization of peroxidase-labelled cholera toxin by neuroblastoma cells. However, their study focused on late events of internalization and the type of surface invagination involved in the early stages of endocytosis was not investigated. The present results demonstrate that both the initial binding and subsequent internalization of gold-labelled cholera and tetanus toxin are mediated by non-coated surface microinvaginations, which suggests that the pathway of endocytosis of a ligand may depend on the chemical nature of its receptor. In this respect, it is interesting that gangliosides, due to their localization in the outer leaflet of the membrane bilayer, may not interact directly with the clathrin coat on the cytoplasmic side of the membrane. The present observations are also consistent with the notion that coated pits may act as molecular filters^{3,26}, concentrating some specific membrane components^{1,6}, but excluding others^{18,26}. The exclusion of gangliosides, in particular, could correlate well with the reported absence of sialic acid groups from coated pits²⁷.

In conclusion, our observations together with the previous studies on antibody-induced endocytosis of HLA antigens⁷ strongly suggest that there are at least two distinct pathways

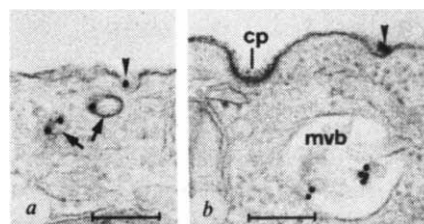


Fig. 2 Internalization of tetanus toxin-gold by cultured liver cells during reincubation at 37 °C for 30 min. Gold particles are present in smooth microinvaginations (arrowheads), in small non-coated cytoplasmic vesicles (arrows) and in a multivesicular body (mvb). There is no labelling of a coated pit (cp). Scale bar, 0.2 μ m.

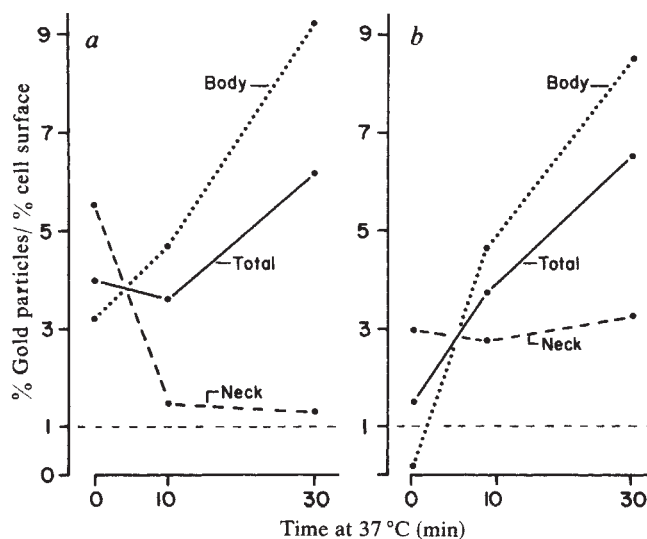


Fig. 3 Temperature- and time-dependent association of gold-labelled cholera (a) and tetanus (b) toxins with non-coated membrane microinvaginations. The time point '0' refers to cells fixed immediately after incubation at 2 °C. The ratio as defined in Table 1 was estimated for the microinvaginations as a whole, and also separately for the neck and body regions. The neck was defined as the region of membrane running from the most constricted part of the invagination up to the flat surface of the plasma membrane, and the body was the portion below the constriction. The neck was found to correspond to 3.4% of the total cell surface length and the body to 5.5%.

for internalization of surface-bound ligands, one involving coated pits and the other involving non-coated microinvaginations.

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Conversion of a gonadotropin-releasing hormone antagonist to an agonist

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Gonadotropin-releasing hormone (pyroGlu¹-His²-Trp³-Ser⁴-Tyr⁵-Gly⁶-Leu⁷-Arg⁸-Pro⁹-Gly¹⁰ amide, GnRH) stimulates pituitary luteinizing hormone (LH) release. An antagonist, D-pyroGlu¹-D-Phe²-D-Trp³-D-Lys⁶-GnRH (GnRH-Ant), binds to the pituitary GnRH receptor and inhibits GnRH-stimulated (10^{-9} M) gonadotropin release from pituitary cultures ($IC_{50} = 2 \times 10^{-7}$ M). GnRH-Ant has no measurable agonist activity at concentrations up to 10^{-6} M. The presence of the D-Lys⁶ both affords protection against proteolysis and includes an amino group which may be used for derivatization without loss of receptor-binding activity. Formation of the GnRH-Ant dimer by cross-linking of the (lysyl) amino groups of two molecules with ethylene glycol bis(succinimidyl succinate) (EGS) results in a GnRH-Ant dimer joined by a 12–15 Å chain. As the amino terminus on GnRH-Ant is blocked, leaving the D-Lys⁶ amino group the only reactive group¹, the reaction of EGS with GnRH-Ant does not lead to larger polymers. Like the parent compound, this dimer is purely an antagonist. We now show, however, that when antibody (AB) to D-Lys⁶-GnRH (which cross-reacts with GnRH-Ant) is incubated with excess dimer, a product is formed which consists of a divalent antibody with a GnRH-Ant dimer attached to each arm: AB-((GnRH-Ant)-EGS-(GnRH-Ant))₂. In contrast to the parent compounds, this conjugate is an agonist, stimulating LH release from pituitary cultures. Our results suggest that a pure antagonist becomes an agonist when it is capable of bringing two receptor molecules within a critical distance, d ($15 \text{ Å} < d < 150 \text{ Å}$). The data indicate that formation of the receptor microaggregate itself is sufficient to stimulate a trans-membrane response system.

Figure 1 shows the ability of GnRH-Ant to inhibit GnRH-stimulated LH release from pituitary cell cultures. GnRH-Ant was an effective inhibitor of stimulated LH release; alone it has no detectable agonist action at concentrations up to 10^{-6} M. Figure 2 shows the elution pattern (G-25 column chromatography) of (1) authentic ¹²⁵I-GnRH-Ant, (2) EGS+GnRH-Ant dimerization reaction mix and (3) the EGS+GnRH-Ant

dimerization mix in which the cross-linking volume was 10 times greater than in (2). Although complete separation of the monomer and dimer was not possible, the leading edge (see bracket in Fig. 2) consisted of fractions of GnRH-Ant dimer which contained <0.5% monomer. To provide evidence for the formation of a dimer, EGS cross-linking was performed in a 10-fold greater than normal incubation volume (a condition which would not favour the two second-order reactions required for the formation of the GnRH-Ant dimer). This resulted in an elution pattern in which a smaller percentage of the total GnRH-Ant was cross-linked by EGS.

We next analysed the activity of AB-((GnRH-Ant)-EGS-(GnRH-Ant))₂. In this conjugate, one molecule of each GnRH-Ant dimer is attached to each antigenic binding site and is therefore unavailable to the receptor. The other molecule of each dimer is available to the receptor, although it is bound to

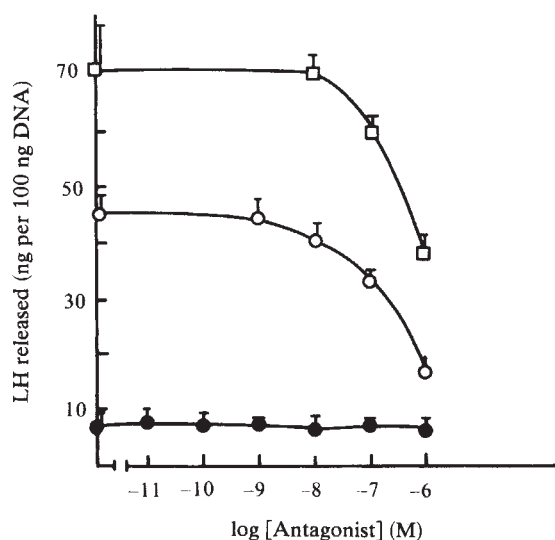


Fig. 1 Antagonist activity of GnRH-Ant in the cell culture bioassay: 2-day pituitary cell cultures² were incubated with 10^{-8} M (□), 10^{-9} M (○), or no (●) GnRH at the indicated concentration of antagonist. After 5 h incubation, the medium was assayed for LH by radioimmunoassay (RIA) using rat anti-LH antisera (LH-4), highly purified rat LH for labelling (LH-I-4) and rat LH standard (LH-RP-1), which were made available by a grant from the NIAMDD Hormone Distribution Office (prepared by Dr A. Parlow). The assay was performed as previously reported², except that a second antibody was used to separate the bound and free ¹²⁵I-LH. The intra-assay variance was 7% and the inter-assay variance 12%. As indicated, LH release was normalized for slight differences in cell numbers in different wells by DNA determination¹⁵.

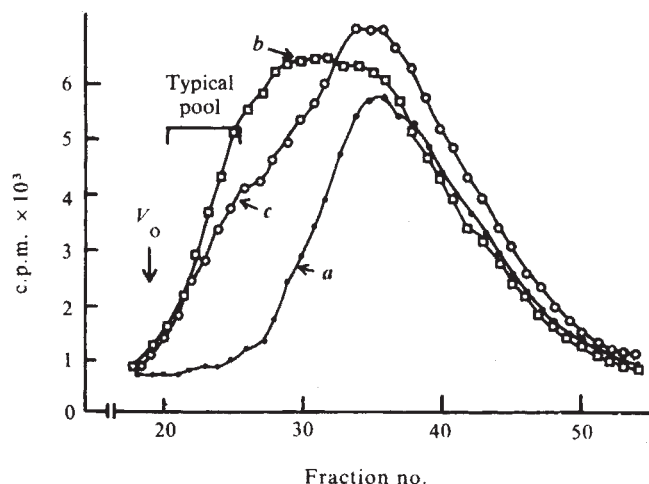


Fig. 2 Elution profiles of ^{125}I -GnRH-Ant (a), the EGS-GnRH-Ant-containing dimerization reaction mixture (showing a typical pool of the purified dimer (b) and the identical reaction conducted in a 10-fold greater volume (c). GnRH-Ant (prepared by J.M.S., batch LR307/29-39) was iodinated using chloramine T¹⁶. Free ^{125}I and unlabelled hormone were removed from the GnRH-Ant by CM-cellulose column chromatography, as described previously except that the labelled analogue was eluted from the column with 200 mM sodium phosphate, pH 4.5. Maximum binding (assessed by binding with excess GnRH antiserum (no. 9113), prepared and used as in ref. 7) ranged from 89 to 92% in different preparations and specific activity (assessed by self-displacement assay with pituitary plasma membrane fragments¹⁷) was $\sim 1 \text{ mCi } \mu\text{g}^{-1}$. The product was stored in the elution buffer at 4°C for up to 2 weeks. At that time, >90% of the hormone (in different preparations) was intact as judged by electrophoresis¹⁷. Ten nmol of GnRH-Ant in 10 μl of 10 mM sodium phosphate, pH 8.5, and 20 μl (~ 0.05 – $0.15 \text{ } \mu\text{Ci}$) of ^{125}I -GnRH-Ant in 200 mM sodium phosphate, pH 8.7, were combined with 5 nmol of EGS (Pierce Chemical, Illinois) in 1.5 μl of dimethyl sulphoxide (DMSO, redistilled from Fisher). The EGS was stored in the dark at -70°C and was dissolved in DMSO immediately before use. As EGS has 2 mol of reactive groups per mol, the reactive groups were present in equimolar concentration with the lysyl ϵ -amino in GnRH-Ant. After 1 h at 18°C , the reaction was terminated by dilution with 270 μl 40% ethanol, 10 mM sodium phosphate, pH 8.5, and immediately applied to a G-25 column ($10 \times 260 \text{ mm}$) previously equilibrated in the ethanolic buffer. Positions of eluting substances were determined by radioactivity and confirmed by RIA (using antibody 9113 at final titre of 1:7,000, radioiodinated GnRH and authentic GnRH-Ant standard).

the antibody via the 15 Å chain and the other molecule of GnRH-Ant. The product thus consists of two molecules of GnRH-Ant available to the receptor although separated from each other by $\sim 150 \text{ Å}$. Figure 3 shows the ability of column fractions containing AB-((GnRH-Ant)-EGS-(GnRH-Ant))₂ (the 'conjugate' which elutes in the V_0) to stimulate LH release from pituitary cultures. In contrast to GnRH-Ant alone, the conjugate displayed agonist action, evoking release of $9 \pm 1.0 \text{ ng LH per } 20 \text{ } \mu\text{l}$ of medium compared with $0.8 \pm 0.1 \text{ ng LH per } 20 \text{ } \mu\text{l}$ medium for basal release and $13.6 \pm 0.9 \text{ ng LH per } 20 \text{ } \mu\text{l}$ of medium released in response to 10^{-7} M GnRH during a 5 h period. Stimulated release was blocked by 2 mM EGTA (in the presence of 'normal' 1.25 mM extracellular Ca^{2+}) and by 5 μM Pimozide (which behaves as a calmodulin antagonist in this system²). In addition, neither the antibody alone (Fig. 3) nor the GnRH-Ant dimer alone stimulated measurable release ($0.9 \pm 0.1 \text{ ng LH per } 20 \text{ } \mu\text{l}$ medium). The reduced pepsin AB fragment (monovalent AB) conjugated with GnRH-Ant dimer did not stimulate release, indicating the requirement for bivalency to produce this effect.

GnRH occupancy of its receptor stimulates pituitary gonadotropin release by a calcium-mediated mechanism³ involving calmodulin^{2,4}. Occupancy leads to patching, capping and internalization⁵, although neither internalization of the releasing

hormone nor large-scale patching and capping of the hormone-receptor complex are required for stimulation of LH release^{6,7}. The results reported here support the view that formation of a microaggregate of the GnRH receptor is sufficient to transduce the signal across the plasma membrane and to elicit cell responses if the separation distance between ligands is increased to a critical point. Thus, although both agonists and antagonists may bind to similar sites on the receptor, the latter is unable to produce the active functional unit. The GnRH-Ant dimer possesses only a 15 Å separation which appears to be inadequate to bridge receptor binding sites; so it cannot stimulate release. The added length due to the antibody indicates that the 'correct' bridge length is $\sim 150 \text{ Å}$.

It is unlikely that the releasing hormone itself bridges two receptors because it is neither multivalent nor sufficiently long. A more likely possibility is that receptor occupancy by an agonist alters the receptor structure so as to increase the probability of microaggregation. For example, occupancy (by an agonist) might increase mobility of the receptor in the plasma

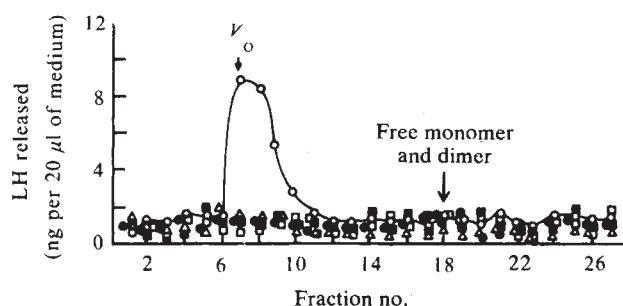


Fig. 3 Effect of column fractions containing AB-dimer conjugate on LH release from pituitary cell cultures. 120–160 ng (GnRH-Ant)₂-EGS collected from the leading edge of the G-25 column described for Fig. 2 (which contained <0.5% of the free GnRH-Ant, or EGS-GnRH-Ant monomer complex, estimated from other column eluant patterns) were lyophilized in a glass tube. Rabbit anti-D-Lys⁶-GnRH (antiserum no. 5111 prepared by immunization with D-Lys⁶ coupled to bovine serum albumin (BSA) with glutaraldehyde) was added without additional purification in an amount sufficient to bind 8–10% of the dimer. The AB was added in a final volume of 50 μl (with Medium 199 containing 0.3% BSA and 2 mg NaN_3). The reaction was carried out for 10 h at room temperature and then for 2 days at 10°C . Immediately before the cell bioassay, the solution was applied to a 12 ml calibrated G-75 column ($10 \times 260 \text{ mm}$) equilibrated in Medium 199/BSA. Twenty drop fractions ($\sim 300 \text{ } \mu\text{l}$) were collected and brought to 1.0 ml with Medium 199/BSA. The conjugate elutes in the V_0 . Where monovalent antibody is specified, the reduced¹⁸ pepsin fragment¹⁹ of antibody 5111 was substituted for intact antibody in the conjugation described above. In this case ammonium sulphate-fractionated IgG was dissolved in 50 mM acetate buffer, pH 4.5, and digested with pepsin (Worthington Biochemical) at an enzyme/substrate ratio of 1:100 at 70°C for 50 min. Digestion was stopped by neutralization with 1 M Tris-HCl, pH 8.0 and 0.1 M NaOH. Precipitated material was removed by centrifugation and 2-mercaptoethanol was added to the supernatant at a final concentration of 0.75 mM. The solution was maintained at room temperature for 1 h, then cooled in ice-cold water. An equal volume of 0.75 mM iodoacetamide (previously degassed and cooled at 4°C) was added and triethanolamine added to maintain the pH at 8.0 ± 0.2 . After 1 h the solution was dialysed against three (2 h each) 100 vol changes of 0.9% NaCl then lyophilized. This product was >95% cleaved as judged by the inability to precipitate ^{125}I -D-Lys⁶-GnRH with protein A. Cell cultures (described in Fig. 1 legend) were incubated with the aliquots from the column fractions in a final volume of 1.0 ml of Medium 199 containing 0.3% BSA. The conjugate ($n = 7$, $\circ$) stimulates LH release (by RIA as described for Fig. 1) which is blocked in the presence of $10 \text{ } \mu\text{M}$ Pimozide ($n = 3$, Δ), or 2 mM EGTA ($n = 3$, $\blacksquare$). A conjugate prepared with monovalent antibody ($n = 3$, $\square$) or bivalent antibody alone ($n = 3$, $\bullet$) did not stimulate release. Standard errors of the means, which are not shown for clarity, were <15% of the indicated value.

membrane by releasing it from anchoring; alternatively, a conformational change might occur such that complementary binding sites are exposed. It is also possible that occupancy activates enzymes which catalyse the formation of disulphide⁸ or peptide⁹ bonds and thereby link receptors. An antagonist would be viewed as occupying the binding site without effecting these changes.

Microaggregation of plasma membrane receptors seems to be required for the activation of some other plasma membrane-regulated systems. In the case of epidermal growth factor (EGF)-stimulated mitogenesis in 3T3 fibroblasts¹⁰, cyanogen bromide treatment of EGF results in a molecule (CN-EGF) which binds to the EGF receptor but does not elicit mitogenesis. Addition of bivalent EGF antibody, which presumably brings together the occupied receptors, results in activation of mitogenesis. Further, anti-insulin receptor antibodies can stimulate insulin-like functions in target tissues¹¹. Bivalent antibodies are required for this stimulation¹², indicating the importance of a receptor microaggregation in this process also.

Divalent antigens such as EGS-(GnRH-Ant)₂ have been useful in the determination of the bridge length between the binding sites on antibodies¹³ and in the demonstration of the bivalency of IgG molecules¹⁴. The technique described here provides a general means of preparing a bivalent antibody that binds to the active site of a receptor from an antibody which was initially prepared by immunization with the ligand.

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Axon segments sprout at both ends: tracking growth with fluorescent D-peptides

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In the nervous systems of many animals, including vertebrates, isolated axon segments can survive for weeks or even months¹⁻³; they can participate in regeneration^{4,5} and in some cases receive synapses from regenerating axons^{6,7}. Axon segments in cell culture exhibit limited growth⁸⁻¹⁰. Although lengths of axon may grow in more intact systems¹¹⁻¹⁴, direct evidence for this has

been difficult to obtain, partly because of the difficulty of marking isolated axon segments. We have injected fluorescently labelled synthetic D-peptides¹⁵ into identified neurones in the leech central nervous system to mark axon segments. We report here that individual segments can initiate growth at both ends and grow for days, both in organ culture and *in vivo*. These results support the hypothesis that axon growth is not essentially polar and suggest a novel mechanism by which axon segments may assist nerve regeneration.

Cell bodies of pressure (P) sensory neurones¹⁶ were injected with either rhodamine- or fluorescein-conjugated D-peptide¹⁵, which spread throughout the cell (see Fig. 1 legend). The synthetic peptide consists of 12 amino acids in the D-configuration, making it resistant to proteolytic enzymes¹⁵. Two days after injection into cells within cultured chains of ganglia, axon segments 2-4 mm long were produced by crushing or cutting the nerve cord at two sites between adjacent ganglia (Fig. 1). Growth was traced at 1- or 2-day intervals by viewing the preparation with an image-intensified camera and epifluorescent illumination¹⁷ (Fig. 2). At the end of the experiment, preparations were fixed and viewed by conventional fluorescence microscopy (Fig. 3).

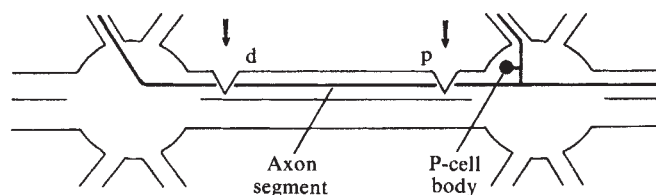


Fig. 1 A schematic diagram of the nerve cord preparation. P-cell bodies were pressure-injected using glass microelectrodes filled with either rhodamine-D-peptide (2% in 0.2 M KCl with 0.2% Fast Green FCF) or fluorescein-D-peptide (2% in 0.025 M Tris-chloride buffer pH 11, with 0.2% Fast Green FCF and 0.1 M KCl). Injections were performed in chains of ganglia pinned to silicone rubber (Sylgard 184, Dow Corning) and maintained in supplemented Leibowitz-15 culture medium^{19,30}. After 2 days, the nerve cord was cut or crushed in two places (arrows) between adjacent ganglia, creating an axon segment with proximal (p) and distal (d) ends.

Axon segments began sprouting within the first day after cutting and continued to grow for at least 6 days (Fig. 2). After longer periods, increased background autofluorescence at the lesion site made it difficult to detect fine branches (or neurites). Typically, fine sprouts emerged from the ends of the severed axon, and branched and grew in several directions within the nerve cord, including backwards along the axon (Fig. 3). Some neurites had at their tips a swelling which seemed to be a growth cone, and small swellings or varicosities were seen at points along the sprouts, features characteristic of growing axons in diverse systems^{9,18}. Of 84 axon segments examined, 42 grew at both ends, 33 grew only at the distal end, one grew only at the proximal end and 8 did not grow detectably. The distal end usually sprouted more profusely than the proximal end. Although individual branches occasionally grew 100 μ m per day for up to 2 days, their average growth rate over a 6-day period was 35 μ m per day or less, a rate comparable to that seen for isolated leech neurones in culture¹⁹. There was no obvious difference in the amount of growth after cuts or crushes, although following cuts those neurites that extended forwards appeared to grow across the cut surface of the nerve cord.

The two cuts or crushes separated axons into three pieces: an axon still attached to the cell body, the axon segment and the piece distal to the axon segment (Fig. 1). All the cut ends sprouted and grew at comparable rates.

To determine whether axon segments can also grow *in vivo*, P-cells were injected *in situ*²⁰ with fluorescent D-peptide. One or two days later, axons were severed by crushing the nerve cord (Fig. 1) and after a further 6 days, chains of ganglia were

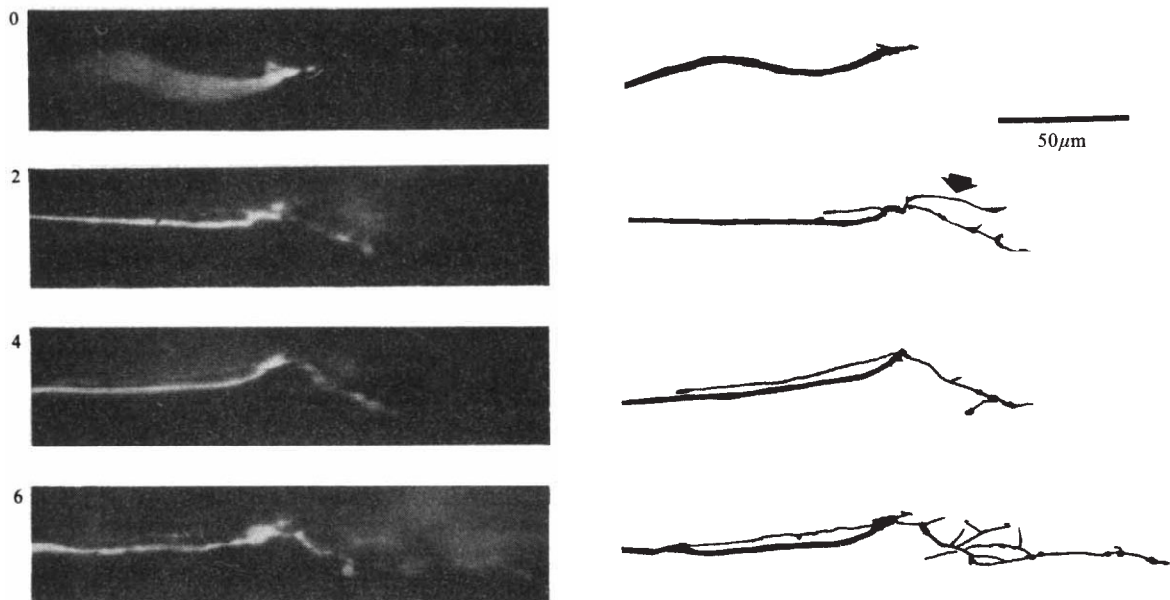


Fig. 2 Growth of the proximal end of an axon segment. Panels on the left are photographs of the video monitor screen at 2-day intervals (number of days indicated at the left) beginning 30 min after the axon was severed by crushing the nerve cord. The shallow depth of field of the objective lens reduced the amount of arborization visible in any single photograph of the monitor screen. Therefore, for each time point, tracings were made from the screen while focussing through the preparation (right-hand panels). The arrowhead indicates a neurite that apparently retracted between days 2 and 4. The preparation was viewed by epifluorescent illumination through a $\times 40$ water immersion objective and suitable filters (Zeiss), in combination with a silicon intensified target (SIT) camera (RCA TC 1040/H), video monitor (RCA TC 1209) and video recorder (Panasonic, NV 8030). To determine the diameter of branches that were detectable with the video system, 2% HRP (Sigma, Type VI) in 0.2 M KCl with 0.2% Fast Green FCF was injected before the fluorescent D-peptide. The tissue was fixed, reacted with diaminobenzidine, and prepared for electron microscopy²². Electron microscopic examination of HRP-filled branches that had been viewed by fluorescence in the live preparation showed that branches of diameter 0.3 μm or less could be seen with the SIT camera.

removed, fixed and examined by fluorescence microscopy. Of 11 segments examined, 5 grew at both ends, 3 grew only at the distal end, one grew only at the proximal end and 2 did not grow detectably. Because the axon segments contained D-peptide, an effect of the peptide injection on growth must be considered. The fluorescent D-peptides we have used do not affect development when injected into single embryonic cells¹⁵, but we found that axons attached to P-cell bodies injected with D-peptide sprouted less than uninjected controls (our unpublished results). This suggests that the growth of axon segments observed here may underestimate the normal growth. Axon segments of P-cells injected with horseradish peroxidase (HRP) sprouted less frequently and profusely than those injected with D-peptide, suggesting that injected HRP inhibits nerve growth. In this context it is interesting that HRP damages frog motor nerve terminals²¹.

The varicosities along neurites of axon segments resemble sites of sensory cell synapses in the leech^{22,23}. Synapses are formed by colchicine-treated segments of frog motor axons during their brief period of sprouting¹⁴, and leech axon segments, which survive for longer periods, may also form synapses.

The present study of leech sensory cells shows that isolated axon segments can initiate growth at both ends and grow for at least 6 days. These results support the idea that growth can be controlled locally within the cell^{24,25}. They also demonstrate that although the axonal cytoskeleton is organized in a polar fashion²⁶ and some²⁷ but not all²⁸ axonal transport is directional, axonal growth need not be intrinsically polar. Because the distal stump can be important in accurate regeneration, the possibility must be considered that sprouting by the proximal end of the stump facilitates contact and recognition between the regenerating axon and its severed stump. The source of materials necessary for growth by axon segments is unknown; they could originate either from within the axon or be transported to it from surrounding glial cells²⁹. Although the mechanism by which axons sprout and elongate is little understood, the present experiments add to the growing body of evidence indicating that

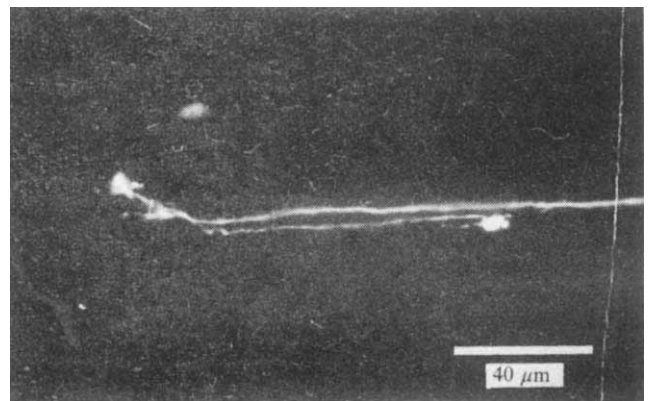


Fig. 3 A montage of fluorescence micrographs of the distal end of an axon segment filled with fluorescein-D-peptide and fixed 4 days after the axon was severed by cutting the nerve cord. The preparation was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 and mounted in glycerol at pH 9.5. Several branches extended forwards and a single neurite, tipped with a large swelling resembling a growth cone, grew backwards along the axon segment.

the axon can have significant functional independence from the cell body.

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Gating currents associated with potassium channel activation

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The voltage dependence of channel-mediated ion conduction in excitable membranes is thought to be due to rearrangement of charged groups or dipoles within the membrane in response to changes in transmembrane potential¹. This charge movement, or gating current (I_g), has been detected in squid giant axons for Na^+ channel activation^{2,3}, but no I_g has been detected which has kinetics similar to those of K^+ channel activation. This is probably due to the relatively slow time course of K^+ channel activation at the temperatures normally used (6–8 °C). We report here that when the temperature is raised to 20–22 °C to increase the rate of channel activation, a new component of I_g is detected which shows kinetics and voltage dependence similar to those of K^+ channel activation at the same temperature. In addition, the net charge moved is consistent with estimates of K^+ channel density. We suggest that this component of I_g is due to the charge movement associated with the rate-limiting step of K^+ channel activation.

Experiments were performed on internally perfused squid giant axons at 20–22 °C using standard voltage-clamp techniques⁴. The internal solution contained either a mixture of potassium fluoride and potassium glutamate (for K^+ currents) or *N*-methylglucamine (NMG) fluoride and NMG-glutamate (for gating currents). The external solution consisted of Na^+ -free Tris-seawater with tetrodotoxin (TTX) added. The holding potential, –60 mV, was chosen to inactivate partially the Na^+ channel gating current⁵.

Figure 1 shows K^+ ionic and gating currents recorded at 21 °C from the same axon after a voltage step to three different potentials. In all three cases the gating current contains two clearly defined components: the fast component, which is the gating current associated with Na^+ channel activation, and the slow component, which is undetectable at 6 °C, and is too slow to be associated with Na^+ channel activation but has a time course similar to the K^+ currents. Figure 2a shows the voltage depen-

dence of the time constants for K^+ channel activation (τ_K) and the slow phase of $I_g(\tau_g)$ for potentials from –30 mV to +30 mV. The two sets of time constants correlate fairly well, indicating that this slow component may be related to K^+ channel activation. Dibucaine was included in the external solution in the gating current measurements to block some of the Na^+ channel gating current. At the concentration used in this study (0.2 mM), dibucaine blocks ~50% of the Na^+ channel I_g without significantly affecting either the K^+ currents or the slow component.

To estimate the amount of charge (Q_s) responsible for the slow I_g , the gating current traces were integrated from the break point in the relaxation to the steady-state level for both ON and OFF gating currents. Although this procedure minimizes any contribution from the fast component, it also ignores any slow charge movement that may have occurred from the start of the voltage step to the start of the integration period. However, as nothing is known about the shape of the early part of the slow I_g , we consider this an adequate method of estimation. We can assign an upper limit to the amount of charge movement that would not be accounted for by the above method by assuming that the slow phase extrapolates exponentially to the beginning of the pulse. When the amount of charge moved is determined in this way, we find that only 10–20% of the charge moved is missed by the original method. Figure 2b shows the voltage dependence of $Q_{s,ON}$ measured by this method: the charge moved is zero at voltages more negative than –80 mV, rises sharply as the voltage becomes more positive and finally saturates at voltages greater than zero. The saturation value of $Q_{s,ON}$ was 508 electronic charges per μm^2 ; the value for all experiments was 500 ± 90 electronic charges per μm^2 ($\pm$ s.d. of 13 determinations). $Q_{s,OFF}$ shows a similar voltage dependence and saturation value. The ratio, $Q_{s,ON}/Q_{s,OFF}$, was found to be 1.1 ± 0.2 ($\pm$ s.d. of 10 determinations), as expected for a true capacitive current.

Figure 2b also shows the voltage dependence of g_K , the K^+ chord conductance (corrected for the non-linearity of the instantaneous current-voltage relation), which is a measure of the fraction of K^+ channels that are in the open state. The voltage dependence of g_K is similar to that of the charge move-

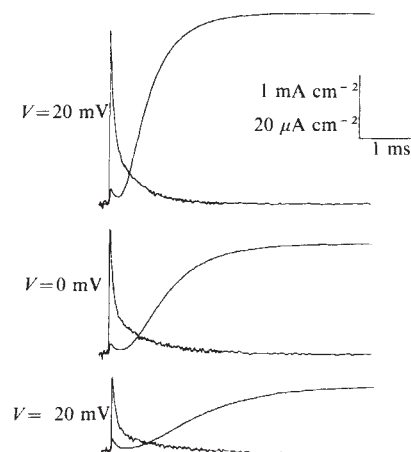


Fig. 1 Potassium (I_K , smooth traces) and gating (I_g , noisy traces) currents recorded at 21 °C in the same axon in response to depolarizations from –60 mV to three different voltages. Leakage and capacitive currents were subtracted using the P/4 technique⁴ with the subtracting holding potential at –110 mV for both currents. I_K records are single sweeps and show gating current at early times. I_g traces are the average of 20 records. The external solution contained 440 mM Tris-HCl, 50 mM CaCl_2 , 0.3 μM TTX pH 7.2. For gating current measurements, dibucaine (0.2 mM) was added to the external solution. Internal solutions were either 66 mM KF, 133 mM K-glutamate, 440 mM sucrose, 10 mM Tris-HCl pH 7.2 (for I_K) or 133 mM NMG-glutamate, 66 mM NMG fluoride, 440 mM sucrose, 10 mM Tris-HCl pH 7.2 (for I_g). Axon JUL211B.

ment, except that the conductance curve is shifted to the right along the voltage axis. Similar behaviour has been observed for the Na^+ channel and is considered evidence for the existence of multiple closed states in voltage-dependent equilibria⁶.

The agreement between the kinetics and voltage dependence of the slow I_g and K^+ channel activation may, however, be fortuitous. K^+ channel activation and Na^+ channel inactivation have similar kinetics and voltage dependence, thus a linkage between Na^+ channel inactivation and the slow I_g will also seem to hold for K^+ channel activation. To test this possibility, we examined the effect of an 8-ms prepulse to -110 mV on the K^+ , Na^+ and gating currents in response to a voltage step to 0 mV. The K^+ currents showed a well-defined Cole-Moore shift⁷ with the activation of channels being delayed markedly (~ 200 μs) when the prepulse was applied (Fig. 3a). The same delay was seen for the slow I_g (Fig. 3b). Na^+ channel inactivation, on the other hand, showed no detectable Cole-Moore shift (Fig. 3c); if it had, then the Na^+ current peak after the prepulse would have shown a definite broadening, but this was not the case.

The results presented here strongly suggest that the slow I_g is caused by the charge movement associated with the rate-limiting step of K^+ channel activation. The time course of both K^+ channel activation and the slow I_g are similar at all voltages examined. In addition, the amount of charge responsible for the slow I_g is consistent with the amount of charge moved as a K^+ channel opens. Using the saturation value of the Q_s - V relationship (~ 500 charges per μm^2) and estimates of the K^+ channel density in squid giant axons (70 per μm^2 ; refs 8, 9), we

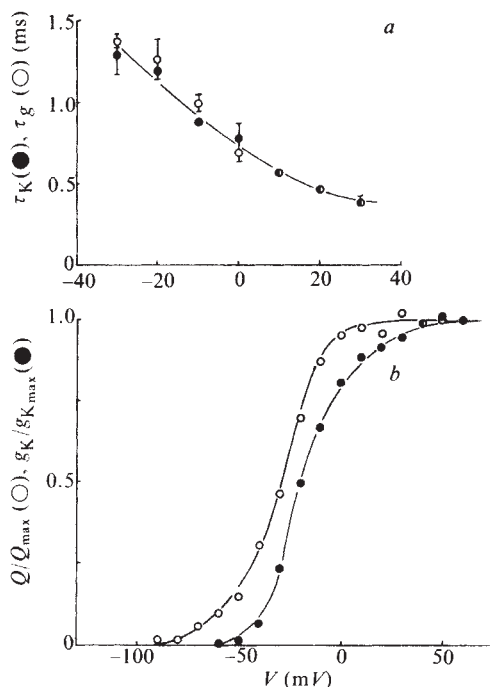


Fig. 2 Correlation of charge movement with K^+ channel activation. Conditions were as described in Fig. 1 legend. *a*, Time constants of K^+ activation, τ_K (●), were determined by fitting the current relaxation after the initial delay to a single exponential. The time constants of the slow I_g , τ_g (○), were determined by fitting the decay of the slow I_g to a single exponential. Each value is the mean $\pm$ s.d. of single determinations from three different axons: JUL211B, JUL221A and JUL221B. *b*, The charge moved during the slow component of ON gating current was determined as described in the text and normalized to the maximum value (508 electronic charges per μm^2 in this case) and plotted as a function of voltage (○). Also shown is the voltage dependence of the K^+ chord conductance corrected for the non-linearity of the instantaneous current-voltage relation and normalized to its maximum value (●). The holding potential was -60 mV and the subtracting holding potential, -120 mV. In all cases the test pulse was preceded by an 8-ms prepulse to -110 mV to make the break point more obvious (see Fig. 3). Axons AUG181A and AUG221A.

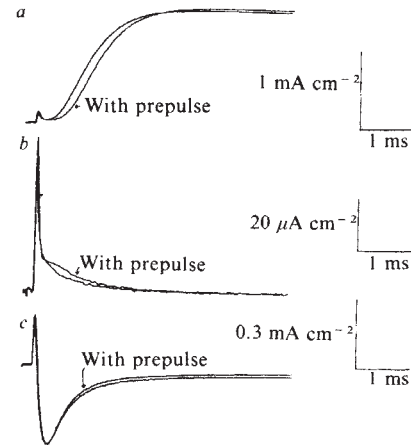


Fig. 3 Effect of a hyperpolarizing prepulse on K^+ (*a*), gating (*b*), and Na^+ (*c*) currents in response to a voltage step to 0 mV. K^+ and gating currents were measured as described in Fig. 1 legend. Trace *c*, which shows Na^+ currents preceded by gating current, was obtained in an external solution containing 90 mM NaCl, 350 mM Tris-HCl, 50 mM CaCl_2 pH 7.2; the internal solution was the NMG solution used for gating current measurements. Where indicated, the depolarizing step was preceded by an 8-ms prepulse to -110 mV. The current trace in *c*, obtained without a prepulse, was multiplied by 1.15 to correct for the difference in peak Na^+ and gating currents. This scaling was done to account for the removal of some resting inactivation by the prepulse. Temperature, 21°C . Axons JUL221B (I_K and I_g) and AUG261A (I_{Na}).

obtain a value of ~ 7 charges per channel. This agrees well with the 5–6 charges per channel expected from macroscopic conductance measurements^{10,11}. It is possible, however, that not all the slow charge movement detected is due to K^+ channel gating currents. There may be contributions from other sources (probably Na^+ channel inactivation), thus the values of Q_s reported here are upper limits. Finally, the slow I_g shows a Cole-Moore shift identical to that seen for K^+ channel activation. This property represents the strongest argument we have that this slow I_g is not a charge movement associated with Na^+ channel inactivation, but actually is the K^+ channel gating current.

Gilly and Armstrong¹² recorded a slow component of I_g at 8°C that correlated with the existence of functional K^+ channels. This charge movement was faster than the development of K^+ currents, which led the authors to suggest that it represented the charge movement associated with a fast step in the activation process. The component described here, however, is not removed by destruction of K^+ channels due to removal of K^+ ions and, furthermore, has kinetics identical to K^+ channel activation. We suggest that the slow I_g described here represents the charge movement associated with the rate-limiting step of K^+ channel activation, while that described by Gilly and Armstrong is associated with a later (the last?) step in the activation process. Such a scheme has been proposed¹³ to explain the bursting patterns seen in single-channel recordings of K^+ channels. Studies of the kinetic behaviour of both these charge movements in response to different pulse protocols should prove valuable in the construction and testing of models for K^+ channel activation, as gating currents can provide information about transitions between closed states that cannot be obtained from conductance measurements.

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Neuropeptide Y—a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide

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The C-terminal α -amide structure is a characteristic feature of many biologically active peptides^{1,2}. Using a novel chemical method for the detection of peptide amides³, we have isolated two naturally occurring peptides, peptide HI (PHI) and peptide YY (PYY), from extracts of porcine intestine and have shown that these peptide amides represent previously unknown biologically active peptides⁴. PHI is structurally and biologically similar to vasoactive intestinal peptide (VIP)⁵ while PYY shows similarities to pancreatic polypeptide (PP)⁶. Preliminary studies indicated that PHI and PYY may both be present in brain as well as in intestine⁴. We report here the isolation from brain extracts of a peptide amide that was thought to be PYY. However, we found that the peptide, while having distinct structural and biological similarities to both PYY and PP, is a previously uncharacterized peptide, which we designate neuropeptide Y (NPY).

NPY-containing fractions were detected from the amounts of tyrosine amide released after treatment of the lyophilized fractions with trypsin or thermolysin using a method described elsewhere^{3,5}. The brain extract and its methanol-soluble peptide fraction were prepared using procedures similar to those described previously⁷; details are given in Fig. 1 legend. The methanol-soluble peptide fraction (1.8 g), obtained from 400 kg of porcine brain, which was also used for the isolation of VIP⁸, was first subjected to gel filtration on a Sephadex G-25 column. The NPY fractions, which also contain secretin and VIP, were pooled and lyophilized and the lyophilized material (690 mg) was chromatographed on CM-cellulose (Fig. 1). The peptide was eluted with 0.02 M ammonium bicarbonate containing 0.025% mercaptoethanol; this procedure yielded a total of 76 mg of NPY fractions which were free from secretin and VIP. These fractions were further purified by HPLC on a reverse-phase silica gel column, μ Bondapak C-18 (Waters); see Fig. 2. The peptide was eluted with 40% ethanol/5 mM ammonium acetate/0.2% acetic acid in isocratic conditions. This step yielded a total of 16 mg of highly purified NPY fractions. A pure peptide preparation was obtained from these fractions by re-chromatography on a HPLC column using a linear gradient elution system consisting of 0.12% trifluoroacetic acid/water and 0.1% trifluoroacetic acid/acetonitrile (Fig. 3).

The peptide preparation was found to be homogeneous by amino acid analysis, N- and C-terminal determinations, and by HPLC and TLC analyses. HPLC showed that the elution profiles of this peptide were different from those of either PYY or PP. Amino acid analysis revealed that the peptide consists of 36 amino acid residues (Table 1). This peptide has tyrosine as its N-terminal residue, tyrosine amide as its C-terminal and five tyrosine (Y) residues per molecule. It was therefore designated neuropeptide Y (NPY).

The intestinal peptide PYY, like neuropeptide Y, is 36 residues long and contains an N-terminal tyrosine and a C-terminal tyrosine amide^{4,6}. PP is also 36 amino acid residues

Table 1 Amino acid compositions and N- and C-termini of the porcine peptides NPY, PYY and PP

Amino acid	No. of residues		
	NPY	PYY	PP
Ala	4.1 (4)	4	5
Arg	4.0 (4)	4	4
Asx	5.0 (5)	2	3
Glx	3.0 (3)	5	5
Gly	1.1 (1)	1	1
His	1.0 (1)	1	0
Ile	2.0 (2)	0	1
Leu	3.0 (3)	4	3
Lys	1.0 (1)	1	0
Met	0.0 (0)	0	2
Pro	3.9 (4)	4	5
Ser	2.0 (2)	3	0
Thr	1.0 (1)	1	2
Tyr	5.0 (5)	5	4
Val	0.0 (0)	1	1
Total no. of residues	36	36	36
N-terminus	Tyr	Tyr	Ala
C-terminus	Tyr-NH ₂	Tyr-NH ₂	Tyr-NH ₂

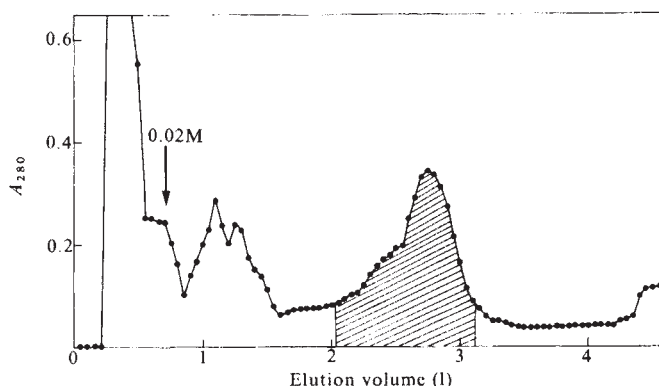


Fig. 1 Ion-exchange chromatography of NPY fraction on CM-cellulose. Porcine brains (400 kg) without cerebellum and pituitary were boiled in water for 10 min, frozen and then extracted with 0.5 M acetic acid (1,000 l) for 24 h. Following filtration, peptides in the filtrate were adsorbed on alginic acid pH 2.7, eluted with 0.2 M HCl and precipitated with NaCl at saturation. The wet weight of peptide precipitate collected was ~800 g. This crude peptide material was dissolved in 16 l of water then 2 vol of ethanol were added, and the pH adjusted to 7.2. After filtration, 3 vol of water were added to the filtrate, peptides in the solution were adsorbed on to alginic acid, eluted, precipitated and collected as described above. This peptide precipitate (80 g wet weight) was dissolved in water and then re-precipitated with NaCl, pH 4.0. The peptide material was suspended in methanol (4 l) containing 0.1% mercaptoethanol and extracted at room temperature for 15 min. After filtration, the methanol extract was neutralized by adding methanolic NaOH solution. The precipitate formed after neutralization was removed by filtration and the pH of the methanol solution readjusted to 2.7 (glass electrode) by addition of methanolic HCl. Peptides in the methanol solution were precipitated by addition of 3 vol of ether. The precipitate was collected by suction filtration and dried under reduced pressure—this yielded 1.8 g of the methanol-soluble peptide fraction. This fraction was subjected to gel filtration on a Sephadex G-25 column in 0.2 M acetic acid and the NPY-containing fractions were pooled and lyophilized. The lyophilized fraction (690 mg) was applied to a CM-cellulose column (5 × 20 cm) equilibrated with 0.01 M ammonium bicarbonate containing 0.025% mercaptoethanol and initially eluted with the same buffer. NPY was recovered after subsequent elution with 0.02 M ammonium bicarbonate containing 0.025% mercaptoethanol. After lyophilization, an aliquot of each fraction was subjected to the chemical assay. The NPY fractions that emerge at 2,050–3,100 ml (indicated by shading) weighed a total of 76 mg after lyophilization.

long and known to have a C-terminal tyrosine amide⁹. However, the amino acid composition of NPY differs from those of PYY and PP (Table 1), but it does have structural similarities to PYY and PP. PYY and NPY, in addition to identical N- and C-terminal amino acids, have equal numbers of Ala, Arg, Gly, His, Lys, Pro, Thr and Tyr residues. Further evidence of sequence similarities was provided by analysis of the tryptic fragments. Treatment of PYY with trypsin yielded five fragments⁶; trypsin treatment of NPY also yielded five fragments, two of which seemed identical to the corresponding fragments of PYY. Moreover, the N-terminal amino acids of all five tryptic fragments of NPY determined by the dansyl chloride method¹⁰ were identical to those of the corresponding fragments of PYY. Furthermore, preliminary amino acid sequence studies of the NPY tryptic fragments using the dansyl-Edman procedure¹¹ strongly suggest that NPY has further sequence similarities to PYY and PP. We have previously reported that PYY and PP are structurally related and together form a new peptide family⁶; NPY may be the third member of this novel peptide family.

PYY and NPY seem to have similarities in biological activities as well as in structure. NPY inhibited secretin-stimulated pan-

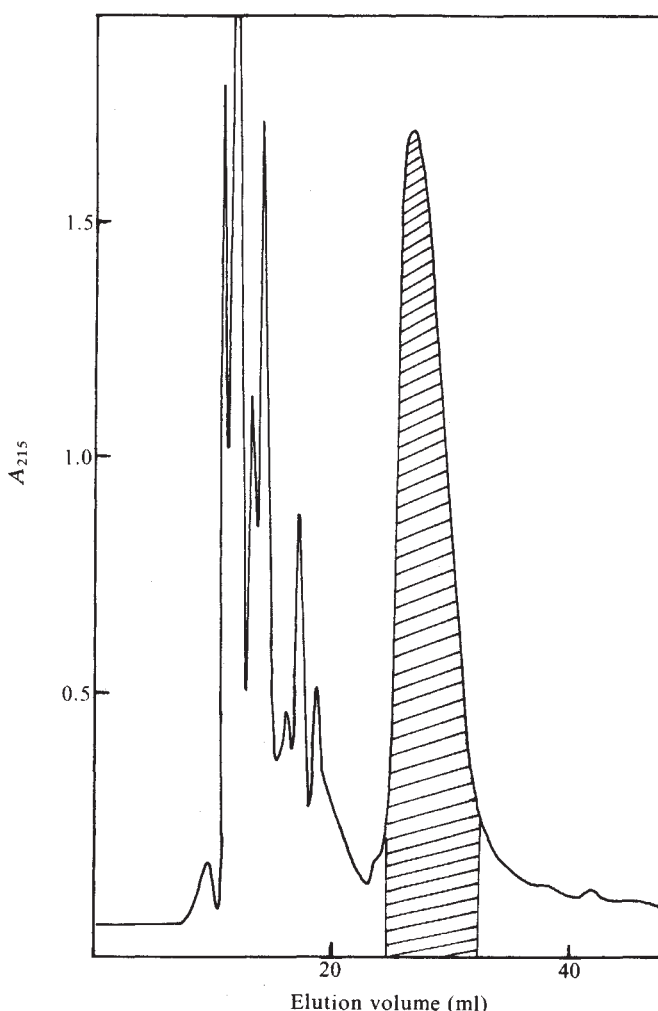


Fig. 2 A typical HPLC elution profile of the NPY fractions (see Fig. 1). The NPY fractions (2 mg per run) were applied to a reverse-phase HPLC column (μ Bondapak C-18, 7.8×300 mm) and eluted with 40% ethanol/5 mM ammonium acetate/0.2% acetic acid at a flow rate of 2 ml min^{-1} in isocratic conditions. The HPLC instrument (Waters) consisted of an injector (U6K), two pumps (M-6000 A), a UV detector (450 variable) and a solvent programmer (Model 660). The peak fractions were collected and concentrated to one-fifth of the original volumes under reduced pressure. After the original volumes were restored by adding water, the fractions were lyophilized and an aliquot of each was subjected to the chemical assay. The NPY peak is shaded.

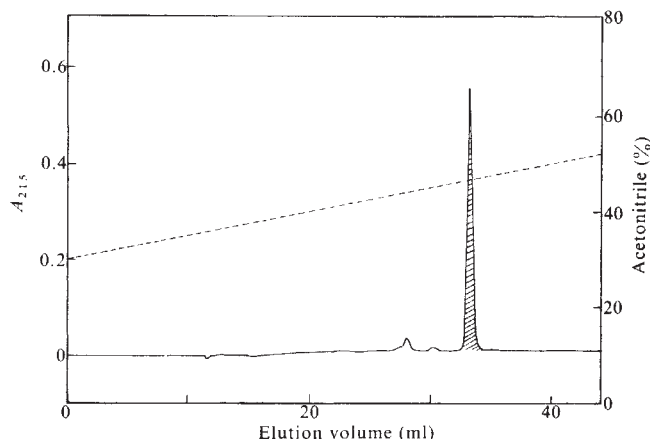


Fig. 3 HPLC elution profile of the NPY peak (see Fig. 2). The NPY fraction ($25 \mu\text{g}$) was re-chromatographed on a HPLC column (μ Bondapak C-18, 7.8×300 mm) using a linear gradient elution system (solvent A, 0.12% trifluoroacetic acid/water; solvent B, 0.1% trifluoroacetic acid/acetonitrile) at a flow rate of 2 ml min^{-1} . The NPY fraction (shaded area) was lyophilized as described in Fig. 2 legend, and then subjected to amino acid analysis and N- and C-terminal determinations.

creatic secretion in the anaesthetized cat (to be published) but was less potent than PYY⁶. PP is also known to inhibit pancreatic secretion in the dog^{12,13} but has no such effect in the cat.

Recently, many gut peptides originally isolated from the gastrointestinal tract have also been found in the central nervous system^{14,15}. However, despite increasing immunochemical evidence for the existence of many such gut-brain peptides, only a few have been isolated from both tissues and characterized with respect to their amino acid sequences. So far, these studies have indicated that the gut-brain peptides from the two tissues have identical amino acid sequences. That of NPY, however, is clearly different from its intestinal counterpart, PYY, although both peptides possess identical N- and C-terminal amino acids and have the same number of amino acid residues. An immunohistochemical study has indicated that PYY may be absent from the brain¹⁶. We have also been unable to detect PYY in the brain, nor has the brain peptide NPY been detected in the intestine by chemical assay and HPLC techniques. It is therefore possible that NPY is found only in nerve cells and PYY only in endocrine cells.

NPY seems to be present in large quantities in the brain, at concentrations even higher than those of VIP¹⁷. Clearly, it will be of interest to determine whether NPY is a neurotransmitter or neurohormone and to discover its physiological role(s) in the central nervous system.

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Acetylcholinesterase forms in fast and slow rabbit muscle

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In the skeletal muscles of vertebrates, the level of acetylcholinesterase (AChE, EC 3.1.1.7.) may be either decreased (in rat) or increased (in chicken¹ and rabbit^{2,3}) after denervation. This enzyme is very polymorphic, however, presenting molecular forms which differ in their cellular localization and physiological regulation. The different AChE molecules may be classified as globular forms (monomers G₁, dimers G₂ and tetramers G₄) and asymmetric or collagen-tailed forms (containing one, two or three tetramers: A₄, A₈ and A₁₂)⁴. The distribution of the collagen-tailed and globular forms along the muscle fibres varies according to the animal species and physiological state: in normal adult rat muscle, the A₁₂ form, which represents the major collagen-tailed form, is localized exclusively in the endplate region⁴⁻⁶, whereas in rat embryo⁷ and human muscle⁸ this form is distributed over the entire muscle fibre. The presence of collagen-tailed AChE has, however, been considered as an indicator of neuromuscular interactions because its appearance during embryogenesis coincides with the establishment of neuromuscular contacts^{6,9} and because, after denervation, the A₁₂ form disappears from rat^{5,6} and chicken^{10,11} muscles. Here we report that this form in fact increases markedly in a slow twitch oxidative muscle of the rabbit after denervation. The regulation of asymmetric—and particularly A₁₂—forms of AChE thus depends dramatically on the species and on the nature of skeletal muscle.

One of the large muscles of the rabbit leg, the semimembranosus, consists of two very distinct and remarkably homogeneous parts^{12,13}: the external region (region I) contains fast twitch type II glycolytic fibres and the internal conoidal bundle (region II) contains only slow twitch type I oxidative fibres (Fig. 1a) (P.V. and F.B., in preparation). Figure 2 illustrates an analysis of the AChE content of endplate-containing and endplate-free segments of these two muscle regions. In region I, the aneural sections contain very little AChE activity, while in region II, the specific activity is about half that of the endplate-containing segments. In both fast and slow muscle regions, however, the collagen-tailed forms are preferentially, or exclusively, localized in the endplate-containing segments (the small proportion which is observed in the aneural samples may be due to the presence of a few endplates which have not been detected). In addition, the globular forms are also concentrated in the neural segments.

The semimembranosus muscle was usually denervated by removing a segment of the corresponding sciatic branch (the rest of the leg remaining mobile). After denervation, we noted a marked atrophy particularly affecting region I of the muscle (Table 1) and, by 2 months, an extensive infiltration of fatty inclusions. Although the red colour of the conoidal region II became paler, the two parts of the muscle remained very distinct. Figure 1b shows the histochemical appearance of the muscle after prolonged denervation. The slow type I fibres of region II are markedly atrophied and keep their acid-stable ATPase properties, whereas the fast type II fibres of region I are more heterogeneous, both in size and ATPase activities.

Table 1 shows that after denervation, AChE activity and acetylcholine receptors (AChR) rose to very high levels, reaching similar values in the two muscle regions at 2 weeks. The receptor level then continued to increase in region I, which became

extensively atrophied. Although the receptor level appeared to decrease in region II, it remained at the high level characteristic of denervated muscle, and it seems unlikely that this interior muscle bundle could be re-innervated while the external mass of the muscle was not. Indeed, we never observed any sign of re-innervation in our experiments.

In denervated muscles, the AChE activity increased markedly, reaching about 10 times the original level in both muscle regions 1 month after the operation. It then continued to increase in region I, but decreased slightly in region II. After very long denervation periods (6 months), the slow conoidal bundle (region II) retained its characteristics, but the fast external part (region I) presented a very profound fatty degeneration, and no longer contained significant AChE activity.

Figure 3 illustrates the changes occurring in the composition of AChE molecular forms in regions I and II after denervation. The proportions of the various molecular forms were estimated from the sedimentation patterns, and used to determine their specific activities (see Table 2). In region I, the smaller globular forms increase after denervation, while the collagen-tailed forms decrease gradually, essentially disappearing 1 month after the operation. They reappear later in significant proportion (Fig. 3).

Region II follows a very different evolution: there is a marked increase of the collagen-tailed forms, both in proportion and in absolute level, which is maintained for at least 2 months.

We obtained very similar results when the sciatic nerve was transected near its root, paralysing the whole leg. Thus, a passive mechanical activity of the denervated muscles does not modify their evolution markedly.

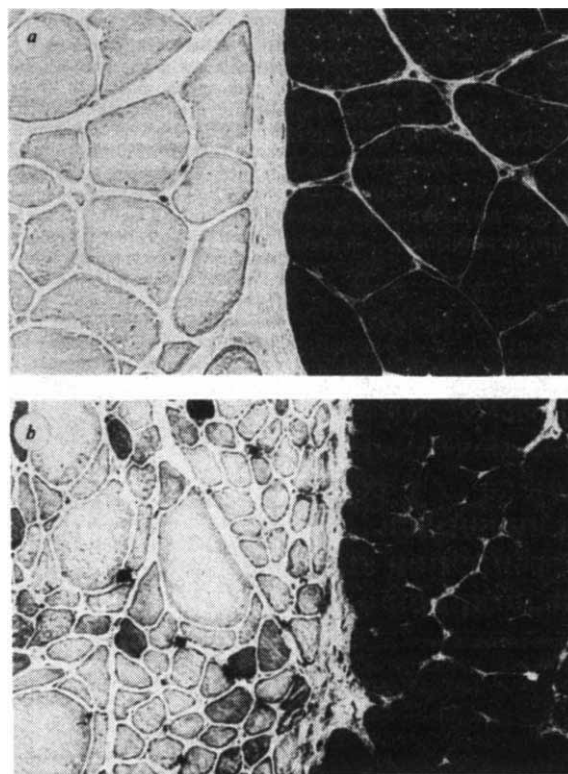


Fig. 1 Histochemical characterization of normal and denervated fast and slow regions of the rabbit semimembranosus muscle. Transverse sections of normal (a) or denervated (3 months) (b) semimembranosus muscle showing the limit between the external rapid part (region I, left) and the internal slow conoidal bundle (region II, right) stained for myofibrillar ATPase at pH 4.15 ($\times 160$). The light-coloured fibres of region I are fast-twitch type II, and the darkly stained fibres of the conoidal region II are slow-twitch type I. Cross-sections 14 μ m thick were cut at -15°C and stained for myofibrillar ATPase according to the method of Padykula and Herman²⁶, modified by Guth and Samaha²⁷. The staining procedure involved 12 min preincubation at 4°C in 1 M formic acid-NaOH buffer (pH 4.15), followed by 60 min incubation (37°C) in 0.1 M barbital buffer, containing 18 mM CaCl_2 and 5 mM ATP (pH 9.4).

The present experiments show that the fast and slow parts of the rabbit semimembranosus muscle maintain their individuality for a very long time after denervation. Muscle types are thus very stable in the absence of innervation. We have in fact shown previously that distinctive characteristics may develop in nerve-free cultures of chick myoblasts obtained from muscles which in the adult become slow or fast¹⁴.

The difference in the evolution of the fast (region I) and slow (region II) parts of the denervated semimembranosus muscle is

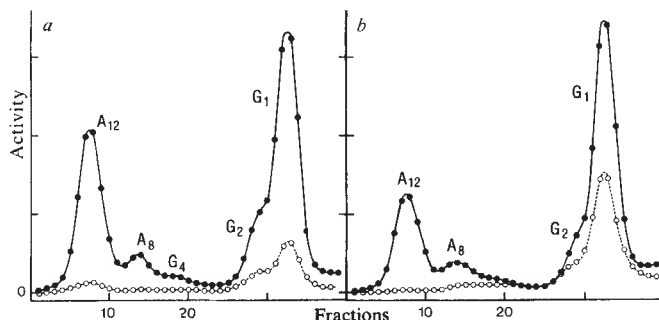


Fig. 2 Sedimentation profiles of AChE in endplate-containing and endplate-free sections of the fast (region I) and slow (region II) parts of the rabbit semimembranosus muscle. *a*, Region I; *b*, region II. ●, Endplate-containing segments; ○, aneurial segments. Small groups of fibres were teased from the two regions of the muscle, and endplates were visualized by the method of Koelle and Friedenwald, modified by Koenig and Rieger²⁸, which does not inactivate AChE. Neural and aneurial segments could thus be dissected. They were homogenized in a glass-Teflon Potter homogenizer, with 10 vols of extraction medium containing antiproteolytic agents (NaCl 1 M, Triton X-100 1%, Tris-HCl pH 7, 10 mM, benzimidazole 1 mM, bacitracin 1 mg ml⁻¹, Zymofren (aprotinin) 25 U ml⁻¹). Aliquots of the extracts were layered on 5–20% w/v sucrose gradients in saline detergent buffer (NaCl 1 M, MgCl₂ 50 mM, Triton X-100 1%, Tris-HCl pH 7 10 mM, bacitracin 0.1 mg ml⁻¹), and centrifuged in a Beckman SW 41 rotor, at 40,000 r.p.m. and 4 °C, for 20 h. *Escherichia coli* β -galactosidase (16S), beef liver catalase (11.3S) and calf intestine alkaline phosphatase (6.1S) were included as internal sedimentation standards, as described previously⁴. The apparent sedimentation coefficients of the AChE forms were approximately 16.2S (A₁₂), 13S (A₈) and 8.5S (A₄) for the asymmetric forms, and 10.5S (G₁), 5S (G₂) and 3S (G₃) for the globular forms. The asymmetric, collagen-tailed forms were characterized by their aggregation properties; they were no longer visible if the gradient was prepared without NaCl. The specific activity of AChE in aneurial segments of region I was very low (0.8 nmol per min per mg protein), compared with that of endplate-containing segment (5.7 nmol per min per mg protein). In the case of region II, the specific activities of aneurial and neural segments were, respectively, 3 and 7.6 nmol per min per mg protein. AChE activity is plotted on an arbitrary scale but the profiles corresponding to the neural and aneurial samples of each region have been normalized so that the activities of each form in the two segments are directly comparable.

particularly remarkable with respect to the collagen-tailed forms of AChE. In region I, the localization and evolution of AChE appear somewhat similar to those previously observed in chicken fast-twitch muscle: the activity is highly concentrated at the endplates in the normal muscle¹⁵, and after denervation there is a concomitant decrease of the A₁₂ form and increase of the small globular forms. In contrast, the dramatic increase in the A₁₂ collagen-tailed form which occurs in the denervated region II of semimembranosus disagrees with previous observations in other species, which suggested that this form disappeared from all skeletal muscles after denervation, even when the overall level of AChE was elevated. This increase shows that the continued presence of the nerve is not necessary for the biosynthesis of collagen-tailed AChE forms, a conclusion reinforced by the reappearance of the A₁₂ form in the fast region (I) of the muscle after prolonged denervation.

In addition, the very high level of A₁₂, and to a lesser extent A₈, in denervated region II indicates that these forms are probably not limited to the original sites of neuromuscular contacts. Indeed, we found that segments from 1-month denervated region II, corresponding to originally endplate-containing and endplate-free regions, contained exactly the same AChE activity with the same pattern of molecular forms, in particular a high proportion of A₁₂ form.

The histochemical analysis of Tennyson *et al.*³ showed that after denervation, the AChE activity increases mostly in the extrajunctional part of the muscle fibres, notably at intracellular sites, and is largely associated with the sarcoplasmic reticulum. It is thus very likely that a fraction of the molecular forms synthesized in such conditions, including the collagen-tailed forms, remain intracellular, associated with the reticulum. One example of such an intracellular localization of collagen-tailed forms has been reported in the case of motor nerves, where these molecules are transported by fast axonal flow¹⁶.

The present observations do not contradict the hypothesis that neuromuscular interactions are required to trigger the biosynthesis of the collagen-tailed forms: Koenig and Vigny¹⁷ found that muscle cells from 13-day-old rat embryos only synthesize the A₁₂ form when cultured in the presence of neurones, but that myoblasts obtained from 18-day-old embryos possess the capacity to synthesize this form by themselves. Rubin *et al.*¹⁸ have also demonstrated an induction of the A₁₂ form in cultures of chick myotubes, by synaptic contacts with cholinergic neurones, while Kato *et al.*¹⁹ found that this enzyme is synthesized in cultures of muscle cells isolated from innervated muscles of 11-day-old chick embryos. These findings

Table 1 Evolution of AChE and acetylcholine receptors in fast (I) and slow (II) regions of rabbit semimembranosus after denervation

		Control muscle	2 Weeks denervated muscle	1 Month denervated muscle	2 Months denervated muscle
Fast twitch semimembranosus region (I)	Total weight of muscle (g)	20.8	11.1	6.0	5.8
	AChE (nmol per min per mg protein)	2.2	13	29.3	62.1
	AChR (pmol per mg protein)	0.01–0.02	0.34	0.47	0.76
Slow twitch semimembranosus region (II)	Total weight of muscle (g)	2.23	1.76	1.10	1.42
	AChE (nmol per min per mg protein)	4.3	15.7	41.6	30
	AChR (pmol per mg protein)	0.01–0.02	0.34	0.19	0.15

The rabbits used were New Zealand white females weighing 2.800–3.100 kg. The weight of the muscle has been normalized for 3 kg body weight. The specific activity of AChE was determined in muscle extracts obtained in a detergent saline buffer containing antiproteolytic agents (Tris-HCl, pH 7 10 mM, EDTA 10 mM, NaCl 1 M, Triton X-100 1%, benzimidazole 1 mM, Zymofren (aprotinin) 25 U ml⁻¹, bacitracin 1 mg ml⁻¹). AChE activity was determined by the spectrophotometric method of Ellman and colleagues²⁴ as described previously⁴ in the presence of 10⁻³ M methopropazine, an inhibitor of nonspecific cholinesterase. The extract was preincubated for at least 1 h in the reaction mixture in the absence of the substrate acetylthiocholine, in order to avoid interference with the reaction of sulphhydryl groups in the extract with 5, 5'-dithio-bis(2-nitrobenzoic acid) (DNTB). We verified that the preincubation of AChE with DNTB did not modify its activity. The activity of the 'nonspecific' cholinesterase (EC 3.1.1.8.) was negligible in the muscle extracts, even after denervation. AChR were assayed by measuring the binding of ¹²⁵I-labelled bungarotoxin (NEN) (10–20 μ Ci per μ g) to a membrane preparation obtained in the following manner. A sample of finely minced muscle was homogenized with a Polytron homogenizer in 10 vol of buffer (Tris-HCl, pH 7.5, 50 mM, EDTA 3 mM, EGTA 1 mM, phenylmethylsulphonylfluoride 0.1 mM, aprotinin (Sigma) 5 trypsin inhibitor units per 1). The homogenate was filtered through a nylon sieve to eliminate the fibrous material. After centrifugation at 20,000g for 30 min, the pellet was resuspended in 5 vol of buffer and aliquots of this particulate suspension were incubated for 2 h at room temperature with a saturating concentration of labelled bungarotoxin (8 nM). In control samples a 100-fold excess of cold erabutoxin was added 1 h before the labelled toxin. After incubation, the suspension was diluted with 2 ml cold buffer, centrifuged and the pellet washed twice with 3 ml cold buffer, before counting. The values given are the means of three determinations, obtained in the same experiment. The low values obtained for the non-denervated muscle were not significantly different in the fast and slow regions. The proteins were assayed by the method of Lowry²⁵ with bovine serum albumin as standard. The values given for AChE and AChR are related to the protein concentrations in the detergent saline extract, and in the particulate fraction, respectively.

Table 2 Evolution of AChE molecular forms in denervated rabbit semimembranosus muscle

		Control muscle	2 Weeks denervated muscle	1 Month denervated muscle	2 Months denervated muscle
Fast twitch semimembranosus region	A ₁₂ (16.2S)	0.8 (36%)	0.6 (4.5%)	— (<0.5%)	1.2 (2%)
	A ₈ (13S)	0.2 (10%)	0.4 (2.5%)	— (<0.5%)	0.7 (1%)
Specific activity (nmol per min per mg total protein) and proportion of total activity	G ₄ (10.5S)	0.1 (4%)	0.5 (5%)	3.2 (11%)	7.6 (12%)
	G ₂ (5S)	0.1 (4%)	0.3 (3%)	1.8 (6%)	3.6 (6%)
	G ₁ (3S)	1.0 (47%)	11.0 (84.5%)	24.2 (83%)	48.4 (78%)
Slow twitch semimembranosus region	A ₁₂ (16.2S)	0.4 (9.5%)	1.9 (12%)	10.8 (26%)	7.2 (24%)
	A ₈ (13S)	0.4 (10%)	0.9 (6%)	3.1 (7.5%)	3.8 (13%)
Specific activity (nmol per min per mg total protein) and proportion of total activity	G ₄ (10.5S)	0.2 (5%)	1.9 (12%)	7.5 (18%)	3.8 (13%)
	G ₂ (5S)	0.1 (2%)	0.3 (2%)	0.7 (2%)	0.8 (2.5%)
	G ₁ (3S)	3.0 (69%)	10.7 (68%)	19.6 (47%)	14.1 (47%)

The proportions of the various molecular forms were estimated from the sedimentation profiles (Fig. 3), and the specific activity of each form was then obtained from the total AChE activity, given in Table 1. Although the minor asymmetric A₄ form represents a significant component in some cases (for example, ~5% in the central slow region), its contribution is generally negligible and is not listed.

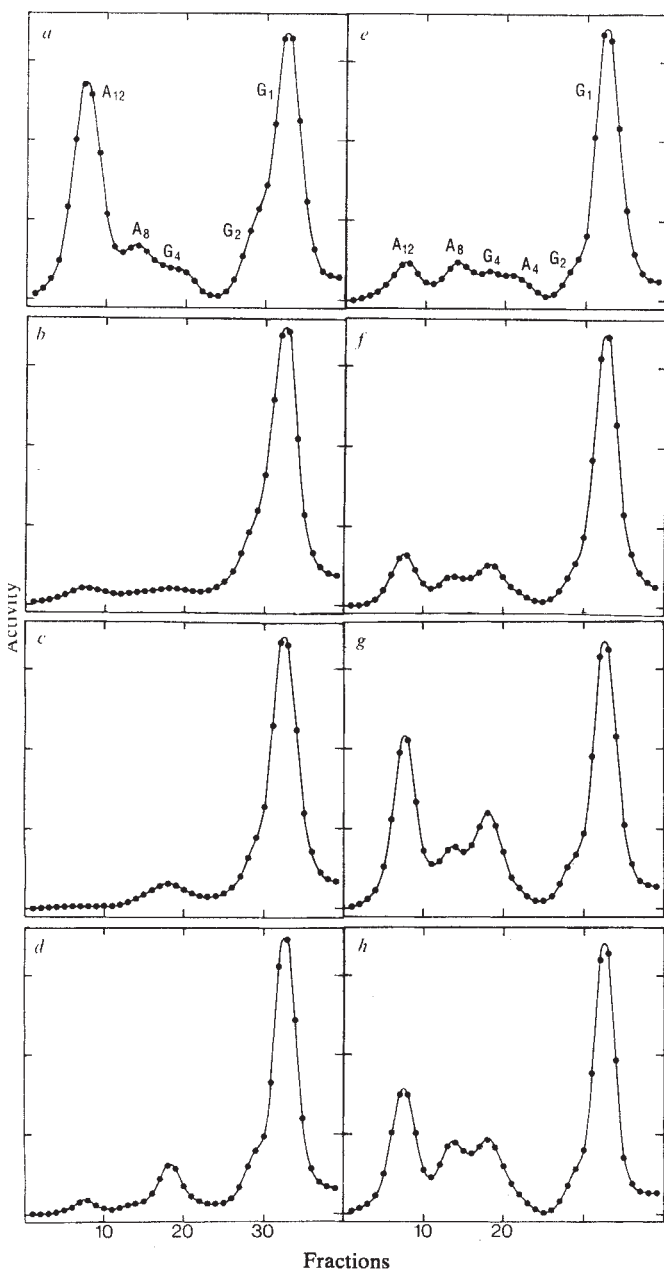


Fig. 3 Sedimentation profiles of AChE in the fast (region I) and slow (region II) parts of the normal and denervated rabbit semimembranosus muscle. *a-d*, region I; *e-h*, region II. The muscle was finely minced with a scalpel; aliquots of the mince were crushed into a powder in liquid nitrogen, homogenized in a Teflon Potter homogenizer and the extracts were analysed as indicated in Fig. 2. The proportions and specific activities of the different forms of AChE are reported in Table 2.

suggest that nerve-muscle interactions occurring *in vivo* may leave permanent instructions allowing the assembly of collagen-tailed AChE in culture. The presence of the A₁₂ form in cultures of a transformed murine muscle line¹⁹ indeed shows that this capacity can be transmitted indefinitely.

It seems likely that the proportion of the different molecular forms of AChE observed in normally innervated muscles *in vivo* corresponds to specific physiological requirements; the different forms possess distinct interaction properties⁴ and their proportions therefore control the spatial distribution of the enzyme within synaptic structures. For example, the ionic interactions of the collagen-tailed forms²⁰ may anchor them in the intersynaptic basal lamina²¹. These forms might be specifically required in neuromuscular junctions at which transmission is effected by impulses of very short duration: in normal adult chickens, the A₁₂ form becomes largely predominant in the phasic, focally innervated posterior latissimus dorsi muscle but disappears in the tonic anterior latissimus dorsi muscle²².

In conclusion, the nerve seems to exert a twofold influence on the muscle: an initial induction effect which allows the synthesis of collagen-tailed AChE and possibly determines the type of fibre—although this can be modified, for example, by reinnervation with a different neurone—and a functional regulation which requires its continued presence. This second effect is probably mediated in part through the muscle's activity²³, perhaps by the intracellular level of cyclic GMP¹⁸. Denervated muscles apparently escape this physiological regulation, producing either increased or decreased levels of AChE, with highly variable patterns of molecular forms, depending on the species, and on the type of muscle.

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A rat monoclonal antibody against mouse α and β interferon of all molecular weight species

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Monoclonal antibodies have proven to be invaluable reagents for the characterization and purification of human interferons (IFN). Hybridoma lines secreting monospecific antibodies against human IFN- α and IFN- β have been isolated, and these antibodies have shown no cross-reactivity between the two interferons^{1–4}. Murine interferons, when analysed by polyacrylamide gel electrophoresis, have two major migration patterns, one in the 28,000–35,000 (28–35 K) molecular weight (M_r) region and the second in the 22 K region^{5–8}. Based on some homology of the N-terminal amino acid sequence with human interferons, the 28 K and 35 K species are classified as mouse IFN- β and the 22 K species as mouse IFN- α ⁹. Minor species have also been described but their relationship to IFN- α and IFN- β is unknown^{10,11}. We have now established a stable hybridoma line secreting rat antibodies to mouse virus-induced interferon. The monoclonal antibodies are of the IgG class, with binding activity to all the different M_r species, as demonstrated in an enzyme-linked immunosorbent assay (ELISA) and by affinity chromatography. In addition, the antiviral activity of all the M_r species was neutralized by the antibody but the neutralizing activity was much weaker than the binding activity. This indicates the presence of a common antigenic site for all mouse α and β interferon species.

The interferon used for immunization was elicited with Newcastle disease virus (NDV) in monolayer cultures of Swiss mouse C-243 cells as described elsewhere¹⁰. Purification was performed by two-step affinity chromatography on poly(U)-Sephacrose and anti-interferon antibody-coupled Affigel-10 columns. The antibody used for this purification consisted of the globulin fraction of serum from a goat immunized with NDV-induced C-243 cell mouse interferon. The specific activity of the purified interferon was 2.4×10^9 U per mg protein (all interferon units are expressed as international reference units) and its electrophoretic purity was assessed on 15% polyacrylamide slab gels in the presence of SDS in a Tris-glycine buffer system⁸. The biological activity of interferon preparations was determined by measuring the protection from the cytopathic effect of vesicular stomatitis virus on L929 cell monolayers.

An 8-week-old female rat of the LOU strain received 5×10^7 U of purified interferon in complete Freund's adjuvant subcutaneously (s.c.) and intraperitoneally (i.p.). This corresponds to 50 μ g of interferon if calculated with a specific activity of 2.4×10^9 U (ref. 8). A second set of injections (s.c. and i.p.) with 2.5×10^7 U (25 μ g) of purified interferon was given 3 weeks later in incomplete Freund's adjuvant. After 3 weeks the animal received 0.5×10^7 U (5 μ g) of purified interferon i.p. on 3 consecutive days. The rat produced serum antibodies against interferon, with a neutralizing titre of 1/8,000 as measured against 4 interferon units. It was killed 2 days after the last

injection and spleen and mesenteric lymph nodes were aseptically removed. The cells were fused with LOU-derived 210RCY3-Ag1.2.3 rat myeloma cells¹² according to the protocol of Köhler and Milstein¹³ modified by Fazekas *et al.*¹⁴. After fusion, the cells were cultured in Falcon Microtest plates at a density of 8×10^5 spleen cells per well on mouse peritoneal macrophage feeder layers in hypoxanthine-aminopterin-thymidine medium; 960 wells were seeded. Supernatants of growing hybridoma colonies were tested for binding activity using an ELISA. Microelisa plates (Dynatech, M 129B) coated with purified interferon were filled with 50 μ l of hybridoma supernatant and kept at 37 °C for 30 min. After washing, horseradish peroxidase-conjugated rabbit anti-rat immunoglobulin preparations (Cappel Laboratories) were used as indicator antibody in 1:200 dilution in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) and 1% Tween 20. After 30 min incubation at 37 °C the wells were washed and for the colour reaction, 100 μ l of 25 μ g *o*-phenylenediamine per ml of 10 mM phosphate buffer, pH containing 0.02% H_2O_2 (30%) were added. The reaction was terminated after 20–30 min with 50 μ l of 3M HCl and absorption was read at 492 nm. Of the 960 wells seeded, 250 were positive for hybridoma growth, and the supernatants of 17 of these gave a positive ELISA reaction (twice the background reading), but only one (clone C5A) was found to be stable after two subclonings. Cells from wells containing supernatant active in ELISA were immediately subcloned by limiting dilution and supernatants from subclones tested. In this way a stable hybridoma line secreting monoclonal antibodies binding to electrophoretically pure interferon was established. The antibody belongs to the rat IgG class, as determined by immunoprecipitation, gel filtration and electrophoresis on 15% polyacrylamide gel in the presence of 0.1% SDS and 1% β -mercaptoethanol. The secretion of antibodies was checked regularly and the cell line found to be stable for over 8 months.

For mass production of antibodies, adult male rats (LOU) were injected i.p. with 2 ml of Pristan mineral oil and 1 week

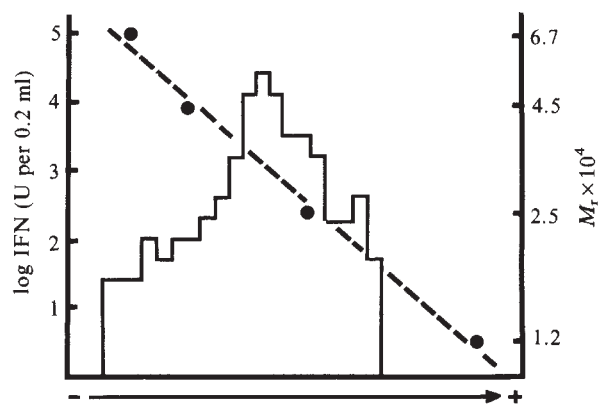


Fig. 1 Electrophoretic profile of interferon activity eluted from polyacrylamide gels after affinity chromatography on monoclonal antibodies fixed to Affigel-10 (Biorad). To prepare the column, ammonium sulphate-precipitated antibodies derived from 50 ml ascites fluid was coupled to the Affigel in 100 mM phosphate buffer, pH 7, at 4 °C. Free binding sites were saturated with ethanolamine and the column washed with a cycle of 100 mM phosphate buffer pH 7 and then with a cycle of 100 mM citrate buffer, pH 2.2. Interferon purified on poly(U)-Sephacrose (7.7×10^5 U) was applied to the column. No interferon activity was recovered in the flowthrough. After a washing step, the bound interferon was desorbed with citrate buffer, pH 2.2. The peak fractions were pooled, dialysed against 125 mM Tris buffer, pH 6.8, containing 1% SDS then concentrated on an Amicon PM10 membrane and boiled with 1% β -mercaptoethanol for analysis on a 15% polyacrylamide gel in the presence of 0.1% SDS. Molecular weight markers consisted of BSA (67,000), ovalbumin (45,000), chymotrypsinogen (25,000) and cytochrome *c* (12,500). For the determination of interferon activity, 2-mm slices were cut and eluted overnight at 4 °C.

Table 1 Neutralization of the antiviral activity of the different M_r species of murine interferon

$M_r(\times 10^3)$	log IFN titre	
	Control	+ Antibody
65	3.28	2.68
35	5.38	4.78
28	4.48	3.88
22	3.28	2.98
15	2.38	2.07
Unfractionated IFN	5.88	5.28

Interferon purified on poly(U)-Sephacrose⁸ was electrophoretically separated on 15% polyacrylamide gels in the presence of 0.1% SDS and 1% β -mercaptoethanol. 1-mm slices of the gel were eluted overnight in 0.2 ml of Tris-SDS 0.1%. The antiviral activity of the eluates was determined in the presence or absence of 2.5 μ l of antibody-containing ascites fluid at each dilution of the samples and expressed as log₁₀ of the interferon titre. Comparable results were obtained when the antibody was prepared from supernatants of hybridoma cultures. The results of four different neutralization experiments indicated no significant difference in the degree of neutralization of the various M_r species. Supernatants of myeloma cultures, concentrated and treated like the hybridoma supernatants, were without effect.

later with $5\text{--}10 \times 10^6$ hybridoma cells, also i.p. One to two weeks later ascites fluid was recovered from the peritoneal cavity and IgG purified by ammonium sulphate precipitation, sometimes followed by gel filtration on Sephacryl-S300. The interferon-binding activity in ELISA from the ascites fluid was 1,000-fold higher than that from culture supernatants.

When crude mouse interferon, consisting of a mixture of all M_r forms, was tested in the presence of purified monoclonal antibody, the antiviral activity was significantly reduced. However, the neutralizing titre was considerably lower than the binding titre, since an antibody preparation giving a positive ELISA reaction at a dilution of 1/12,800 completely neutralized 4 U of mouse interferon only at a dilution of 1/32, or lower.

Anti-interferon antibodies from culture supernatant were retained by protein A-Sepharose in binding conditions via the Fc part of the IgG molecule. Crude interferon was applied to the column after extensive washing, antigen-antibody complexes were desorbed. The elution pattern of interferon activity desorbed at pH 3, after electrophoresis on 15% polyacrylamide gel in the presence of SDS, shows that the protein A-Sepharose coated with the monoclonal antibodies retained both IFN- α (22 K) and IFN- β (28–35 K). Insufficient interferon was used in this experiment to allow detection of the 65 K and 15 K species. Additional evidence for binding to all interferon species was then obtained by affinity chromatography on monoclonal antibodies bound to Affigel-10. In these conditions, there was complete retention of NDV-induced C-243 interferon and the bound interferon could be desorbed by lowering the pH with 0.1 M Na-citrate buffer. Because the major α and β interferon species are present in about equivalent amounts in our crude C-243 cell interferon preparation, as shown by neutralization with anti-mouse IFN- β serum, total retention by the column is indicative of affinity for all M_r forms. This was confirmed by an electrophoretic analysis of the interferon desorbed from the column which showed that the major M_r forms were present in the desorbed material (Fig. 1). The minor 15 K species was not recovered from the gel, but affinity of the antibody for this species was demonstrated in the following experiments.

To determine the capacity of the antibody to neutralize all the different mouse interferon species, a preparation of NDV-induced C-243 interferon, prepared as described previously¹⁰, was purified and concentrated on poly(U)-Sephacrose. An aliquot of the peak fraction, with a titre of 2.4×10^7 U, was dialysed overnight against sample buffer (0.125 M Tris pH 6.8, β -mercaptoethanol 1%, SDS 0.1%) and after boiling for 2 min, electrophoresed on a 15% polyacrylamide slab gel as described previously⁸. The gel was cut into 1-mm slices which

were eluted overnight in 0.2 ml of Tris-SDS 0.1% per slice. Titration of the interferon activity of all fractions in the presence or absence of the monoclonal antibody revealed the presence of the two major peaks, one at 28–35 K (murine IFN- β) and one at 22 K (murine IFN- α) and the two minor peaks of 65 K and 15 K. The antiviral activity of all the fractions was reduced by a factor of two to four in the presence of the antiserum: the results for the peak fractions are summarized in Table 1. Affinity of the antibody for all the M_r forms was then confirmed in an ELISA assay, in which a 50- μ l aliquot of each fraction was left to adsorb to Microelisa plates (Dynatech M129) overnight at room temperature. The reaction was then carried out as described above, using antibody obtained from ascites fluid. A positive reaction was scored in all the wells that had received the fractionated interferon, from 65 K to 15 K down.

All previously described monoclonal antibodies against human interferon show specificity for either IFN- α or IFN- β . Recently, a monoclonal rat antibody against mouse IFN- β has been obtained that did not bind mouse IFN- α ¹⁵. Therefore, it is rather surprising that the first monoclonal antibody we obtained after immunizing a rat showed no specificity in binding to a particular mouse interferon species but instead binds to all of them. Antigenic differences between mouse IFN- α and IFN- β have been described by Yamamoto and Kawade¹⁶, who immunized rabbits against the fast- and slow-moving components of NDV-induced mouse L-cell interferon. The antisera they obtained showed some cross-reactivity as measured by binding of interferon to immobilized antibody columns and the authors attributed this to possible cross-contamination of the interferons used for immunization.

The results with the monoclonal antibody presented here suggest that the antisera of Yamamoto and Kawade may well have contained cross-reacting antibodies. Tryptic digests of the high M_r species (40 K, corresponding to mouse IFN- β) and the low M_r species (24 K, corresponding to mouse IFN- α) of mouse L-cell interferon have revealed the presence of a common polypeptide structure¹⁷ and regions of homology have also been found in the amino acid sequence of human α and β interferons¹⁸. As indicated by the results of the binding and neutralization experiments with the rat monoclonal antibody that we have obtained, at least one antigen site is shared by all M_r forms of mouse interferon. This may be a highly conserved region of some importance for the activity of the different interferon species.

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Monoclonal antibodies against unique *I*-region gene products expressed only on mature functional T cells

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The immune response (*I*) region of the murine major histocompatibility complex (MHC) regulates specific immune responses against antigens¹ and encodes cell-surface molecules designated as Ia antigens². Ia antigens are generally detected on B cells and macrophages, and have important roles in immunocyte interactions. On the other hand, it has been reported that some T cells and their products (antigen-specific T-cell factors) carry unique *I* region-encoded determinants which are different from B-cell Ia antigens³⁻¹⁰. As T-cell factors are known to mediate T-T and T-B cell interactions¹¹, elucidation of such unique *I*-region determinants on T cells is obviously important for understanding the regulatory cell interactions of the immune response. However, this has long been hampered by the lack of reagents specific for the determinants and due to the small number of T cells expressing them. To overcome this difficulty, cell hybridization techniques have been used to develop T-cell hybrids which continuously express *I*-region products, and monoclonal antibodies specific for these determinants have been produced. Here we report the production of monoclonal antibodies which detect a unique set of *I*-region determinants expressed only on functional subsets of T cells and T-cell hybridomas.

The rationale used in this study was based on our previous findings that antigen-specific augmenting (T_aF) and suppressor (T_sF) factors produced by immune splenic T cells of mice carry *I*-region determinants encoded in the *I*-A and *I*-J subregions of the MHC^{6,9}. Both these determinants are distinct from B-cell Ia antigens with respect to molecular size, composition, antigen-specific regulatory function and serological cross-reactive pattern. In addition, they possess antigen-binding sites with idiotypes and immunoglobulin variable region determinants¹². These properties suggest that the Ia antigens associated with

T-cell factors may have prototypic structures entirely different from those of conventional B-cell Ia antigens, which consist of α and β polypeptides¹³.

We have obtained T-cell hybrids which produce antigen-specific T_aF and T_sF with *I* region-encoded determinants^{14,15}. Such hybrids are apparently ideal tools for screening monoclonal antibodies reactive to putatively unique T-cell Ia antigens. We therefore produced monoclonal antibodies which react specifically with these determinants expressed on T-cell hybrids. Spleen cells of A.TH (*H*-2¹²) mice immunized with A.TL (*H*-2¹¹) lymphoid cells were fused with P3-X63-Ag-8-653, a 8-azaguanine-resistant plasmacytoma of BALB/c mouse origin. Strains A.TH and A.TL differ only in the *I* and *S* regions of the MHC, thus spleen cells of immune A.TH mice should contain B cells specific for *I*-region products of *H*-2^k haplotype. The fused cells were selected in hypoxanthine-aminopterin-thymidine medium, and were cloned by limiting dilution. The culture supernatants of cell lines were screened for antibody activity against a T-cell hybrid (FL10) of A/J mouse origin, which had been shown to produce T_aF specific for keyhole limpet haemocyanin (KLH) associated with a unique product of the *I*-A subregion of *H*-2^a (ref. 14).

The supernatants of 31 out of 201 clones were reactive with FL10 in a microcytotoxicity assay and in fluorescence-activated cell sorting (FACS) analysis of two fusion experiments. Those clones which stained parental thymoma, BW5147, and Ia-negative T-cell hybrids were excluded. They were further screened with spleen cells of nude C3H mice (*H*-2^k), and only those which did not react with B cells and macrophages were selected. Eleven clones were finally established using the criteria that their antibodies reacted with FL10 but not with BW5147 and *H*-2^k B cells (Table 1).

The monoclonal antibodies were tested further with enriched antigen-binding T cells of A/J (*H*-2^a) mice prepared by adsorption of KLH-primed splenic T cells to antigen-coated plastic dishes¹⁶; A/J mice produce augmenting T cells expressing *I*-A^k but are unable to make *I*-J⁺ suppressor T cells on immunization with KLH⁹. All the antibodies reactive to FL10 were, in fact, strongly cytotoxic for the enriched KLH-binding T cells of A/J but were unable to kill non-antigen binding T cells (Table 1). They were also unable to kill normal splenic and thymic T cells of both A/J and C3H, suggesting that the proportion of T cells expressing these *I*-region determinants is too small to be estimated by the usual cytotoxicity test. The

Table 1 Cytotoxicity of monoclonal antibodies for *I*-A⁺ T-cell hybridoma FL10 and lymphocyte subsets

Monoclonal antibody	Isotype	% Lysis of targets by complement-dependent cytotoxicity				
		FL10	Thymocytes	C3H nu/nu spleen cells	KLH-binding splenic T cells of A/J	KLH-non-binding splenic T cells of A/J
11L9	IgM	52	0	1	21	0
2L2	IgM	65	0	1	38	0
3L1	IgM	44	0	0	21	0
7L114	IgM	19	0	0	36	0
14P	IgM	33	0	0	15	0
21L27	IgM	40	0	0	16	0
34L5	IgM	25	0	0	20	0
88L7	ND	31	0	0	21	0
119L2	IgG	27	0	0	12	0
148L4	IgG	32	0	0	22	0
164L1	ND	14	0	0	25	0
A.TH anti-A.TL		60	0	80	30	0

Screening of monoclonal antibodies using the T-cell hybrid, FL10, and KLH-binding T cells. Culture supernatants of hybrid clones were tested for reactivity to various preparations of normal cells, as well as to the Ia-positive T-cell hybrid, FL10, by the dye exclusion cytotoxicity test. 20 μ l of culture supernatant were mixed with the same volume of cell suspension (2×10^6 ml⁻¹) and incubated at room temperature for 15 min. After washing, 20 μ l of selected rabbit complement were added, and the mixture was incubated at 37 °C for 30 min. Per cent lysis was calculated using the formula $(a/b)/(100-b) \times 100$ where a = percentage of cells stained by Trypan blue after incubation with the culture supernatant and complement, and b = percentage of cells stained after incubation with complement alone. KLH-binding T cells of A/J mice were prepared from spleen cells of KLH-hyperimmunized A/J mice. Spleen cells were first layered onto plastic dishes coated with purified anti-mouse immunoglobulin to remove B cells. The T-cell fraction thus obtained was incubated at 37 °C in plastic dishes coated with KLH. The cells adhering to the dishes were recovered by rigorous washing with chilled medium, and were used as KLH-binding T cells. Nonadherent T cells were used as control. Isotypes of the monoclonal antibodies were determined by double immunodiffusion with purified anti-isotype antibodies. ND, not determined.

T-cell specificity of the monoclonal antibodies was confirmed by absorption of culture supernatants with A/J splenic B cells obtained by treatment with a rabbit anti-brain antiserum and complement (C): after absorption, the cytotoxic activity for FL10 remained intact, whereas absorption with unseparated spleen cells removed the antibody activity (Table 2). This result indicates that some T but not B cells express the *I*-region determinants detected by our monoclonal antibodies.

Haplotype and *I*-subregion specificities of the monoclonal antibodies were determined by absorption with splenic T cells of different strains (Table 2). Absorption with spleen cells of A/J, A.TL, B10.BR ($H-2^k$) and B10.A(4R) eliminated the ability to kill FL10, whereas absorption with A.TH and B10.S ($H-2^s$) cells failed to do so. Cytotoxicity for FL10 was not removed by absorption with B10 ($H-2^b$) spleen cells, except for one (21L27) which showed a definite cross-reactivity with the $H-2^b$ haplotype. These results indicate that the antibody specificity is directed primarily at the product of the *I*-A subregion of $H-2^k$. All the monoclonal antibodies were entirely refractory in their cytotoxicity for virus-producing T lymphomas such as EdG2 ($H-2^d$), EL-4 ($H-2^b$) and AKSL-2 ($H-2^k$), which excludes the possibility that they are antiviral antibodies.

Having established that these monoclonal antibodies consistently react with I -A⁺ FL10, we performed experiments to determine whether they are actually detecting *I*-region determinants selectively expressed on certain functional T-cell

subsets. We used two different methods: (1) the KLH-primed C3H ($H-2^k$) helper T cells were treated with monoclonal antibody and complement, and the cells were co-cultured with syngeneic B cells primed with a hapten, 2,4-dinitrophenyl (DNP), to examine the effects on carrier (KLH)-specific helper T cells; and (2) the KLH-primed splenic T cells were treated with monoclonal antibody and complement, and KLH-specific T_HF was extracted from the cells to assay residual T_HF activity by addition to the *in vitro* secondary anti-DNP antibody response⁹.

Figure 1a illustrates the ability of some of the monoclonal antibodies to eliminate the helper activity of carrier-specific splenic T cells. Figure 1b demonstrates that many of these monoclonal antibodies are also effective in eliminating T_HF-producing cells. In some cases, the response was greatly suppressed, suggesting that the selective killing of T_HF producer resulted in the predominance of remaining suppressor effects. A well-defined monoclonal antibody against a B-cell Ia antigen (anti-Ia.2) was found to be ineffective in eliminating both the helper and augmenting T cells. The ability of the monoclonal antibodies to remove helper and augmenting T cells did not always correlate; as some of the antibodies could kill only T_HF producer, there was an apparent reduction in the net helper activity as demonstrated in Fig. 1a. It was further confirmed that immunoadsorbents of at least three of the monoclonal antibodies (2L2, 3L1 and 14P) tested were able to absorb FL10-derived T_HF (data not shown).

The results indicate that there exist *I*-region-encoded determinants expressed only on helper and augmenting T cells as detected by our monoclonal antibodies. These determinants seem entirely different from conventional Ia antigens of B cells and macrophages, as none of our monoclonal antibodies reacted with these cell types. It is of interest that the determinants are the products of genes mapped to the *I*-A subregion, the chromosomal segment which was defined originally by the presence of the *Ir-1* gene¹ and later by the *Ia-1* locus controlling the B-cell Ia antigen². As none of our antibodies react with B cells, we presume that the *I*-A subregion carries genes which are selectively expressed on augmenting and helper T cells. In addition, we have developed several hybridomas producing antibodies against at least three discrete molecules on suppressor and helper (T_H) T cells controlled by genes in the adjacent *I*-J subregion (A. Kurata *et al.*, unpublished results). Thus, we suggest that there exists a set of multiple loci tandemly arranged on the *I*-region chromosomal segment which is expressed only on mature functional T cells. As some of these *I*-region gene

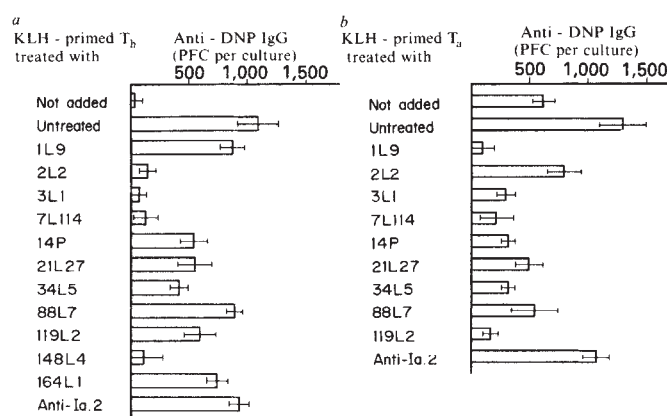


Fig. 1 a, Effect of monoclonal antibodies on the KLH-specific helper T cell (T_H). DNP-primed B cells were obtained from spleen cells of DNP-KLH-primed mice after treatment with rabbit anti-brain antibodies and complement. They were co-cultured with KLH-specific syngeneic helper T cells obtained by incubating KLH-primed spleen cells in plastic dishes coated with rabbit anti-mouse immunoglobulin (anti-MIg). The KLH-primed splenic T cells were treated with monoclonal antibodies (~0.1 µg antibody ml⁻¹) and complement before the co-culture with B cells. Anti-DNP IgG-forming cells (plaque-forming cells, PFC) were counted 5 days after the start of cultivation. Note that treatment with some of the antibodies (2L2, 3L1, 7L114 and 148L4) resulted in complete loss of helper activity. Other monoclonal antibodies show variable degrees of effects on helper activity. b, Effect of monoclonal antibodies on the KLH-specific augmenting T cell (T_A). T cells of KLH-primed C3H mice were obtained by incubating the spleen cells in anti-mouse immunoglobulin-coated plastic dishes. They were then treated with monoclonal antibodies and complement, and the live cells were recovered by centrifugation on a layer of fetal calf serum. T_HF was extracted from the cells by repeated freezing and thawing followed by ultracentrifugation at 40,000g for 1 h. The cell-free extracts corresponding to 3 × 10⁶ original T cells were added to the test culture of DNP-KLH-primed spleen cells 2 days after the start of cultivation. This procedure was found to be suitable for detecting the T_HF activity without much effect due to T_SF⁷. Anti-DNP IgG PFC were estimated 5 days after the onset of culture. Note that all monoclonal antibodies had an effect on the T_HF activity, except anti-Ia.2 which is specific for the B-cell Ia antigen. The suppression of the antibody response in some cases is probably due to the predominant suppressor effect after selective elimination of augmenting T cells (see text).

Table 2 *I*-A subregion assignment of two monoclonal antibodies by absorption with strains of various haplotype

Spleen cells used for absorption	% Lysis of FL10 by	
	14P	1L9
A/J ($H-2^a$)	28	24
A/J (isolated B cells)	<10	<10
A.TH ($H-2^{12}$)	22	ND
A.TL ($H-2^{12}$)	34	30
A.TL ($H-2^{12}$)	<10	<10
B10.BR ($H-2^k$)	<10	<10
B10.A(4R) ($H-2^{h4}$)	<10	<10
B10.S ($H-2^s$)	30	20
B10 ($H-2^b$)	32	31

Culture supernatants of two representative clones, 1L9 and 14P, were absorbed with various doses of spleen cells from strains of different haplotypes, and were tested for their residual cytotoxic activity against FL10. The results given here are those obtained by absorption with 1–5 × 10⁶ cells for 100 µl of culture supernatant. B cells were isolated from A/J spleen cells by treatment with anti-brain antiserum and complement. Note that absorption with I -A^k spleen cells invariably eliminated the cytotoxicity of monoclonal antibodies for FL10, whereas spleen cells having a haplotype other than I -A^k were unable to absorb the antibody activity. One other monoclonal antibody (21L27) cross-reacted with $H-2^b$. Some others showed cross-reactivity with I -J^k and I -E/C^k products; B10.A(3R) and B10.A(5R), as well as B10.A(4R), could absorb the anti-FL10 activity. These will be described elsewhere.

products on T cells were originally defined as a component of antigen-specific T-cell factors¹¹, that is, putative antigen receptors of T cells, biochemical and genetic studies of the molecules reactive to our monoclonal antibodies are required to define the T-cell unit responsible for antigen recognition. Furthermore, our results cast new light on the organization and function of MHC in relation to the genetic regulation of various T-cell functions. The reagents described here should be useful for re-examining the antigen receptors of T cells and the molecular mechanism of MHC-restricted cell interactions.

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Structural homology of a macrophage differentiation antigen and an antigen involved in T-cell-mediated killing

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Two distinct murine cell-surface differentiation antigens, Mac-1 and LFA-1 (lymphocyte function-associated antigen 1), are compared here and shown to be related at the molecular level. Mac-1, defined by the M1/70 rat anti-mouse monoclonal antibody (MAb), is expressed on macrophages, natural killer cells and 50% of bone marrow cells, but not on B or T lymphocytes¹⁻³. In contrast, the LFA-1 antigen, defined by the M7/14 rat anti-mouse MAb, is expressed on B and T lymphocytes and 75% of bone marrow cells, but not on thioglycollate-induced peritoneal exudate macrophages^{4,5}. MAb blocking studies suggest that LFA-1 participates in T-lymphocyte-mediated killing and T-lymphocyte antigen-specific responses^{4,5}. Mac-1 and LFA-1 have α -polypeptide chains of 170,000 and 180,000 molecular weight (M_r), respectively, and both contain β polypeptides of 95,000 M_r . This similarity prompted us to investigate their relationship. Mac-1 and LFA-1 have distinct cellular distributions, MAb-defined antigenic determinants and α -subunits, but have highly homologous or identical β -subunits as shown by tryptic peptide mapping. Moreover, they share some common antigenic determinants recognized by a polyclonal antiserum. Cross-linking studies show that in each antigen the subunits are noncovalently associated in $\alpha_1\beta_1$ structures. Mac-1 and LFA-1 comprise a novel family of two-chain leukocyte differentiation antigens.

The distinct cellular distributions of the Mac-1 and LFA-1 antigens are emphasized by their reciprocal expression on

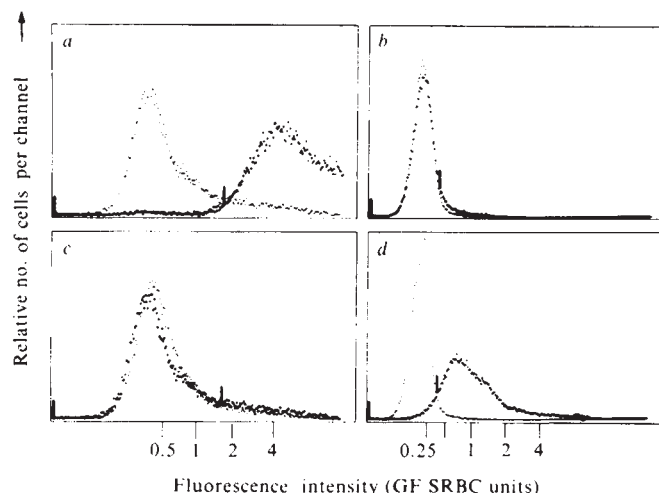


Fig. 1 Reciprocal immunofluorescent labelling of peritoneal exudate cell macrophages and spleen cells by M1/70 and M7/14 MAb. Cells ($50\mu\text{l}$, 5×10^7 ml in RPMI-1640, 5% fetal calf serum, 20 mM HEPES, 0.01 M NaN_3) were labelled with an equal volume of either M1/70 (a, b) or M7/14 (c, d) MAb-containing culture supernatants (dark dots) or NSI culture supernatant containing $50\mu\text{g ml}^{-1}$ normal rat IgG as control (dim dots), washed, then labelled with affinity-purified fluorescein isothiocyanate rabbit F(ab')_2 anti-rat IgG absorbed with mouse IgG. Immunofluorescence flow cytometry was performed on a Becton Dickinson FACS II equipped with a Nuclear Data log amplifier which was calibrated with glutaraldehyde-fixed sheep red blood cells (GF SRBC). Four-day thioglycollate-induced PEC (a, c) or spleen cells (d) were scatter gated to exclude red cells and lymphocytes, or red cells, respectively.

spleen cells and thioglycollate-induced peritoneal exudate cell macrophages (PEC) (Fig. 1). Spleen cells are >93% LFA-1 positive but only 6% Mac-1 positive, while exudate macrophages showed no significant LFA-1 expression but are >94% Mac-1 positive. Expression of Mac-1 and LFA-1 on 50 and 75% of bone marrow cells, respectively^{1,6}, shows these antigens also have distinct myeloid distributions, and are co-expressed on a subpopulation of these cells.

Immunoprecipitation also confirmed that Mac-1 and LFA-1 are reciprocally expressed on concanavalin A-stimulated spleen

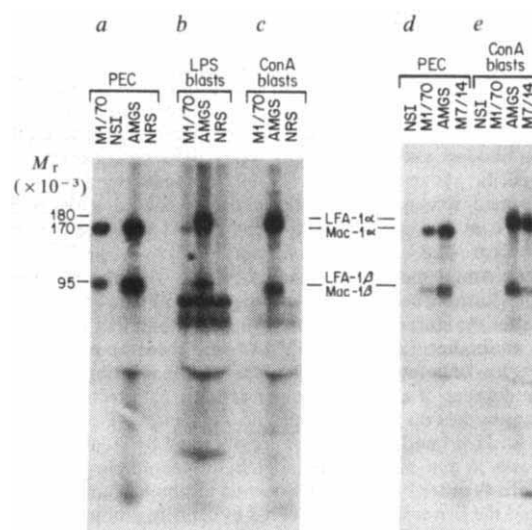


Fig. 2 SDS-PAGE of antigens precipitated from different cellular sources by M1/70 and M7/14 MAb and a polyclonal antiserum. Four-day Con A blasts (c, e), 4-day thioglycollate-induced PEC (>90% macrophages) (a, d) and LPS blasts (b) were prepared, surface labelled with ^{125}I , detergent solubilized, precleared, immunoprecipitated and subjected to SDS 5-15% gradient PAGE and autoradiography as previously described^{1,6}. Two separate experiments (a-c and d, e) with independent cell preparations and SDS-PAGE were carried out. Cell lysates were immunoprecipitated with 25 or $50\mu\text{l}$ of NSI culture supernatant containing $50\mu\text{g ml}^{-1}$ normal rat IgG (NSI) or $1\mu\text{l}$ of normal rat serum (NRS) as controls, $1\mu\text{l}$ of AMGS, 25 or $60\mu\text{l}$ of M1/70 MAb supernatant (M1/70), or $20\mu\text{l}$ of 1mg ml^{-1} M7/14 IgG (M7/14).

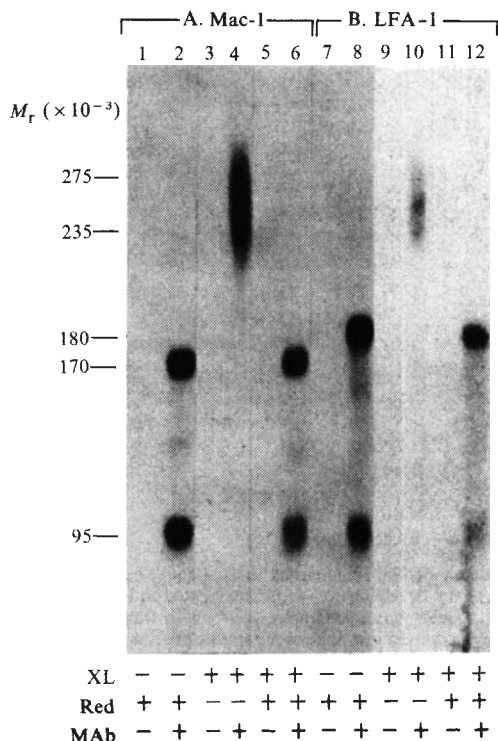


Fig. 3 Cross-linking of Mac-1 and LFA-1. PEC and EL-4 cells were surface labelled with ^{125}I using chloroglycoluril, and Triton X-100 cell lysates were prepared as previously described⁶, except haemoglobin carrier protein was omitted. Lysates were dialysed against 0.1 M NaCl, 0.05 M sodium borate, pH 9, and 800- μl aliquots were either mixed with 16 μl of 5 mg ml^{-1} dithiobis(succinimidyl propionate) (Pierce) dissolved in dimethylformamide, or not treated. After 1 h at 21°C, samples were dialysed against two changes of 0.14 M NaCl, 0.05% NaN₃, 0.01 M Tris HCl pH 7.5. Aliquots were immunoprecipitated with antibodies coupled to Sepharose and subjected to SDS-5% PAGE and autoradiography as previously described⁶. *a*, precipitates from PEC with M1/70 MAb- or normal rat IgG-Sepharose. *b*, Precipitates from EL-4 with M17/4 MAb- (anti-LFA-1, Sanchez-Madrid *et al.*, unpublished) or normal rat IgG-Sepharose. Samples were cross-linked (XL) and either reduced (Red) with 5% 2-mercaptoethanol or treated with 50 mM iodoacetamide before electrophoresis as indicated in the figure. Molecular weights were determined with standards as previously described⁶. Molecular weights >200,000 were determined by extrapolation and should be considered tentative. Cross-linked products were fuzzier than other bands, because the number and position of the cross-links formed affects hydrodynamic properties in SDS-PAGE.

cells (Con A blasts) and PEC, and that the MAb do not cross-react between them. Thus, M1/70 but not M7/14 precipitated material from PEC (Fig. 2*d*), while M7/14 but not M1/70 precipitated material from Con A blasts (Fig. 2*e*). No cross-reaction between M1/70 and M7/14 could be detected, even when autoradiography was prolonged by a factor of five. This was as expected from the differing specificity of the MAb for cells (Fig. 1).

Despite these differences in cellular distribution and antigenic determinants recognized by MAb, Mac-1 and LFA-1 have strikingly similar two-chain structures. The LFA-1 α -chain is slightly higher in M_r than the Mac-1 α -chain, while the β -chains are identical in M_r (Fig. 2*d, e*). Determination of M_r by co-electrophoresis with standards in nongradient SDS gels at three different polyacrylamide percentages showed that the Mac-1 and LFA-1 α -chains are 170,000 and 180,000 M_r , respectively, and the β -chains 95,000 M_r (data not shown). The α - and β -chains are not disulphide linked^{1,6}.

Precipitation by MAb of two distinct polypeptide chains could be because both chains express the antigenic determinant, or because only one chain expresses the determinant but is non-covalently associated with the other chain. To investigate the quaternary structure of Mac-1 and LFA-1, cross-linking experiments were carried out. Detergent-solubilized lysates were cross-linked with the cleavable reagent dithiobis(suc-

cinimidyl propionate) and subjected to immunoprecipitation and SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 3). Instead of α - and β -chains (Fig. 3, lane 2), a cross-linked Mac-1 product of M_r 235,000–275,000 was found (Fig. 3, lane 4), in good agreement with the M_r of 265,000 predicted for an $\alpha_1\beta_1$ structure. Furthermore, the product was composed of α - and β -chains, as shown by reductive cleavage of the cross-links (Fig. 3, lane 6). A similar cross-linked product was obtained with LFA-1 (Fig. 3, lane 10). These results strongly suggest that both Mac-1 and LFA-1 contain α - and β -subunits which are noncovalently associated into $\alpha_1\beta_1$ quaternary structures.

One method of testing for structural homology between two proteins is by immunological cross-reactivity. MAb are not necessarily suitable, as they may recognize unique rather than shared antigenic determinants. Therefore, cross-reactivity was tested with a classical antiserum. A rat antiserum was raised against Mac-1 which had been partially purified from thio-glycollate-induced macrophage membranes by *L. culinaris* lectin affinity chromatography and by MAb immunoadsorbent depletion of two other immunodominant antigens⁷. This anti-macrophage glycoprotein serum (AMGS) potentially precipitated Mac-1 and little or nothing else from PEC (Fig. 2*a, d*). The AMGS also precipitated large amounts of material from lipopolysaccharide-stimulated spleen cells (LPS blasts) and Con A blasts with an M_r identical to that of LFA-1, while this material was not precipitated by M1/70 (Fig. 2*b, c*). The material precipitated by AMGS from PEC and Con A blasts was shown to be identical to Mac-1 and LFA-1, respectively, by co-migration in SDS-PAGE (Fig. 2*d, e*), and by Cleveland peptide mapping (data not shown).

To determine whether precipitation of LFA-1 by AMGS was due to a true cross-reaction of anti-Mac-1 antibodies with LFA-1, or to the presence of antibodies with independent specificities in the AMGS, it was important to test whether the anti-LFA-1 activity could be absorbed with thio-glycollate-elicited macrophages, which are Mac-1⁺ LFA-1⁺. Therefore, AMGS was absorbed with either macrophages or Con A blasts, and the amount of LFA-1 precipitated from Con A blasts was measured by quantitative scanning of autoradiograms (Fig. 4). Despite their lack of LFA-1 expression, macrophages gave

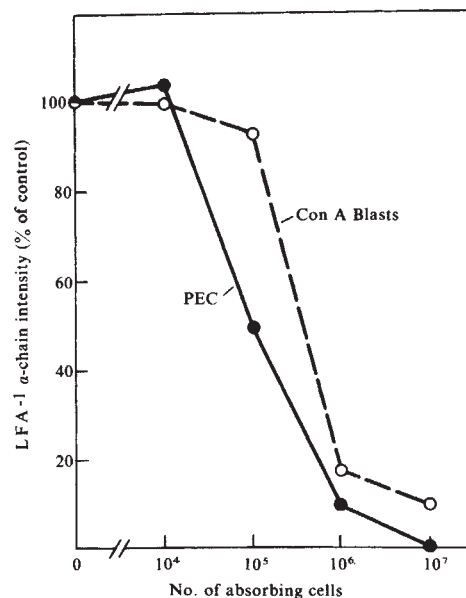
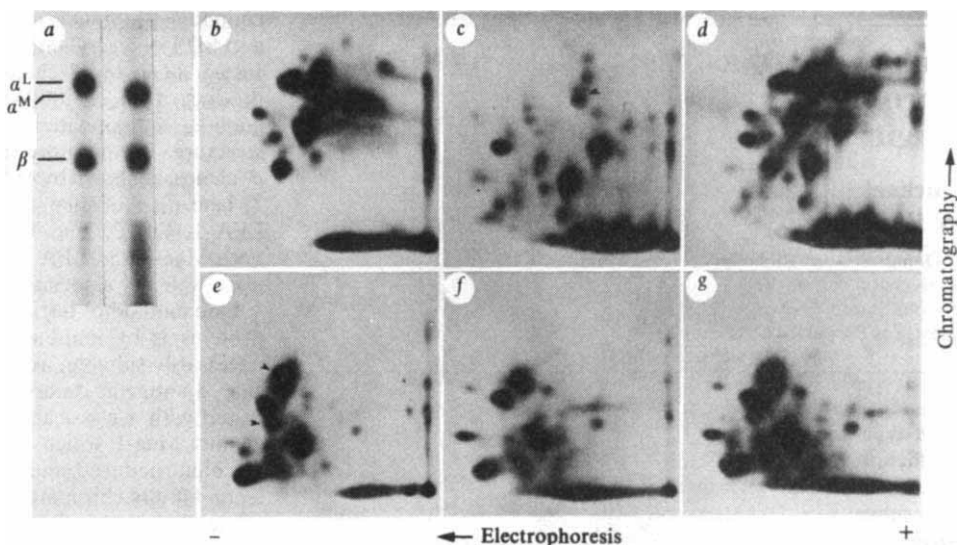


Fig. 4 Absorption by peritoneal exudate macrophages and Con A blasts of the ability of AMGS to precipitate LFA-1 from Con A blasts. AMGS (10 μl of a 1:100 dilution, which gave 70% maximal LFA-1 precipitation) was absorbed with PEC or Con A blasts in 100 μl of phosphate-buffered saline for 2 h at 4°C. Cells were pelleted, and 80 μl of supernatant was used in indirect precipitation of ^{125}I -labelled Con A blast lysates and subjected to SDS-PAGE as described in Fig. 2 legend. After autoradiography with intensifying screens and hypersensitized Kodak XAR film, the film was scanned with a densitometer and LFA-1 α -chain was quantified as peak area.

Fig. 5 Tryptic peptide maps of SDS-PAGE purified LFA-1 and Mac-1 α - and β -subunits. LFA-1 and Mac-1 were purified from EL-4 and P388D₁ cells, as will be described in detail elsewhere. Briefly, EL-4 or P388D₁ detergent lysates in the presence of protease inhibitors were purified by affinity chromatography on M7/14 or M1/70 coupled to Sepharose CL-4B, respectively, and eluted with 20 mM glycine-NaOH, pH 10, or 20 mM triethanolamine, pH 11, respectively, in 0.5% Triton X-100, 1 mM phenylmethylsulphonyl fluoride. Eluates were neutralized and repurified by a second cycle of affinity chromatography. Purified antigens were concentrated and dialysed against phosphate-buffered saline, and 10–15 μ g was iodinated in a volume of 100 μ l with 1 mCi 125 I and 6 μ g chloramine-T (ref. 10) for 1 min, followed by addition in sequence of 6 μ l of 1 mg ml⁻¹ Na₂S₂O₅, 5 μ l of 20 mg ml⁻¹ KI and 5 μ l of 20 mg ml⁻¹ bovine serum albumin. Rat IgG was also iodinated and treated in parallel as a control. After dialysis, the α and β polypeptides were separated by SDS-7% PAGE and the wet gel was autoradiographed for 10 min (panel a). Bands were excised and digested with TPCK-trypsin as described by Elder *et al.*¹¹. Peptide aliquots (5–8 $\times$ 10⁴ c.p.m. in 2–4 μ l) were spotted on 10 $\times$ 11.5 cm cellulose TLC plates (EM Laboratories). Electrophoresis was in 15% acetic acid, 5% formic acid, chromatography was in *n*-butanol/pyridine/acetic acid/water (65:50:10:40), followed by autoradiography. Comparison to the control rat IgG peptides showed that none of the spots were due to free 125 I or simple adducts. b, LFA-1 α -chain; c, Mac-1 α -chain; d, mixture of α -chains; e, LFA-1 β -chain; f, Mac-1 β -chain; g, mixture of β -chains. The arrows in b, c and d are explained in the text.



complete absorption. Furthermore, the macrophages were three-fold more effective than Con A blasts in absorbing anti-LFA-1 antibodies, showing absorption could not be attributed to contaminating lymphoid cells. The greater effectiveness of the macrophages was consistent with our previous findings that macrophages express 16×10^4 Mac-1 sites per cell while Con A blasts express 7.8×10^4 LFA-1 sites per cell^{2,6}. It was concluded that antibodies to Mac-1 cross-react with LFA-1, and hence these molecules are structurally related.

The structural basis for this homology between Mac-1 and LFA-1 was investigated by peptide mapping. Mac-1 and LFA-1 were purified from the P388D₁ macrophage tumour line and the EL-4 T-lymphoma line, respectively, by MAb affinity chromatography, and labelled with 125 I. The α - and β -subunits were separated by SDS-PAGE (Fig. 5a), excised from gels and subjected to peptide mapping. Tryptic peptide mapping showed the Mac-1 and LFA-1 β -subunits share at least 10 tyrosyl peptides, and are thus highly homologous or identical (Fig. 5e–g). One peptide unique to the LFA-1 β -subunit and one increased in intensity (arrows) may be due to carbohydrate processing differences between the EL-4 and P388D₁ tumour lines, rather than to sequence differences. Cleveland peptide maps of LFA-1 and Mac-1 β -chains isolated from Con A blasts and PEC were identical.

The α -subunits had very different tyrosyl tryptic peptide maps (Fig. 5b–d). The α -chains from Mac-1 and LFA-1 showed 17 and 18 unique tryptic peptides, respectively. Co-migration of one peptide (marked by an arrow) was confirmed by a mixing experiment (Fig. 5d). These extensive differences strongly suggest that the Mac-1 and LFA-2 α -chains are products of distinct genes.

The large structural differences in the α -subunits suggest that the unique determinants recognized by the M1/70 and M7/14 MAb reside on these subunits. The β -subunits seem to be immunoprecipitated by virtue of their noncovalent association with the α -subunits. The α - and β -subunits do not appear structurally related, because they showed no tryptic peptide map identities. It seems likely that the common determinants on LFA-1 and Mac-1 revealed by polyclonal anti-Mac-1 antibodies are on the β -chain, although it is possible that the α -chains also cross-react. Definitive proof of where the determinants lie will require testing of antibodies on isolated chains.

Mac-1 and LFA-1 comprise a novel family of leukocyte differentiation antigens which contain noncovalently associated

α and β polypeptides of 170,000–180,000 M_r and 95,000 M_r , respectively. Alternative forms of the α polypeptide can be associated with a common or highly homologous β polypeptide in $\alpha_1\beta_1$ structures. Only two other two-chain leukocyte cell-surface antigen families have previously been described, the major histocompatibility antigens⁸ and immunoglobulins⁹. By analogy with these and other protein families such as the haemoglobins which have shared homologous or identical subunits, it seems likely that the Mac-1 and LFA-1 α -chains are also products of closely linked, homologous genes. The tryptic peptide differences in the α -subunits do not rule out homology, since amino acid sequence homologies of the order of 25–50% would be missed by peptide mapping. The selective expression of the Mac-1 and LFA-1 α -chains in the monocytic and lymphoid lineages is a particularly interesting feature of this differentiation antigen family. The structural relationships between these molecules suggest that the α - and β -chains may mediate specialized and general functions, respectively. LFA-1 plays an important part in antigen-specific T-lymphocyte-mediated killing of tumour cells and in T-helper cell responses^{4,5}. Elucidation of the function of Mac-1 would provide a further comparison with LFA-1, and might provide important insights into the structure–function relationships of the α - and β -subunits.

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Enhanced transformation of human fibroblasts by origin-defective simian virus 40

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Transformation of semipermissive human fibroblasts (HF) by wild-type simian virus 40 (SV40) or SV40 DNA is relatively inefficient compared with SV40 transformation of non-permissive rodent cells¹. Whereas HF transformed with either SV40 or a subgenomic fragment of SV40 (that is incapable of making virions) containing the early region and the origin of DNA replication produce large amounts of free virus DNA²⁻⁵, established human cell lines transformed by SV40 harbour defective virus genomes⁶ that are incapable of initiating virus DNA replication (M. Botchan, personal communication). We have now investigated whether the low efficiency of transformation is directly related to the ability of SV40 DNA to replicate autonomously in semipermissive HF. We have compared the efficiency of transformation of HF by origin-defective SV40 DNA (SV ori⁻) with that of other derivatives containing a functional viral origin of replication, using the calcium phosphate co-precipitation technique⁷. The transforming potential of SV ori⁻ mutants was found to be superior.

The SV ori⁻ mutant is a hybrid DNA consisting of plasmid (pMK16) and the full genome of SV40 DNA less 6 nucleotides at the *Bgl*I site^{8,9}. It is able to transform rat cells as efficiently as the ori⁺ SV40 derivative, but, unlike the latter, is able to transform permissive monkey cells¹⁰. The cell line chosen for the present studies was a human fibroblast designated 'HS74BM' (HF), which is derived from fetal bone marrow and displays a diploid human karyotype¹¹. This cell line is semipermissive for SV40, producing virus at an efficiency of ~1% that of permissive monkey kidney fibroblasts¹². It has been shown to be transformable by DNA transfection with an early region fragment of SV40, albeit at a low frequency⁵.

Initial experiments revealed that the SV ori⁻ recombinant molecule was capable of transforming HS74 as assayed by focus formation in monolayer and colony formation in soft agarose ('anchorage independence'). Transformed colonies express T antigen as detected by immunofluorescence. The actin cable organization of these transformants was consistent with typical SV40 transformants (R. Pollack, personal communication).

Direct quantitative comparison among intact superhelical form I SV40 DNA, plasmid SV ori⁺, plasmid SV ori⁻, as well as among linear form III plasmid SV ori⁺ and plasmid SV ori⁻ is shown in Table 1. Figure 1 shows typical foci. pSV ori⁻ DNA was most efficient in both transformation assays (focus formation and anchorage independence) as well as in the different protocols used after DNA transfection (see Table 1 legend). The results are most striking for the anchorage independence assay, which is considered the more stringent *in vitro* transformation assay¹³. It is also the most stringent for cell viability because it requires colony formation.

This finding strongly suggested that the absence of a functional origin of replication in SV ori⁻ resulted in its enhanced transformation efficiency. However, as plasmid SV ori⁺ DNA had a higher transforming potential than did virion DNA, other possibilities were considered. First, the improved efficiency might be solely due to the presence of adjoining plasmid sequences and/or modification of DNA through propagation in a bacterial host¹⁴. As shown in Table 2, we compared the efficiency of transformation by linearized virion DNA with linear viral DNAs derived from pSV ori⁺ and pSV ori⁻, from which plasmid DNA was removed (lines 1, 3, 4). Again SV ori⁻ was the most efficient. Residual contribution from the bacterial

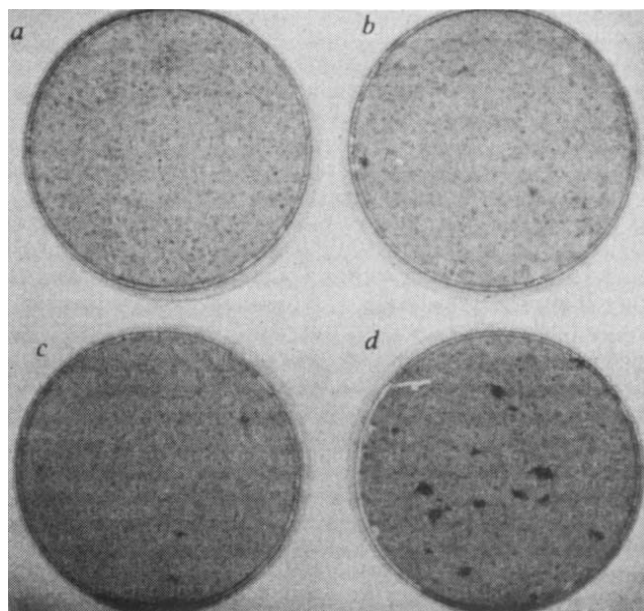


Fig. 1 Giemsa-stained plates of HS74BM 3 weeks post-transfection with SV40 DNA (form I) as described in Table 1 legend. *a*, no SV40 DNA; *b*, SV40 virion DNA; *c*, pSV ori⁺ DNA; *d*, pSV ori⁻ DNA.

Table 1 Transformation of HF by form I and form III DNAs

DNA derivative	<i>In situ</i> foci*	3 Day subcultivation		7 Day subcultivation	
		Foci	Colonies in agarose	Foci	Colonies in agarose
SV40 Virion I	0.5 (0, 1)	0.7 (0, 1, 0, 2)	0.5 (0, 1, 0, 1)	6.0 (4, 8)	0.0 (0, 0)
pSV ori ⁺ I	1.5 (2, 1)	2.0 (2, 0, 2, 4)	2.7 (6, 0, 2)	1.5 (2, 1)	0.5 (0, 1)
pSV ori ⁻ I	3.5 (2, 5)	8.5 (5, 14, 10, 5)	3.3 (2, 2, 6)	10 (10, 10)	6.0 (2, 10)
pSV ori ⁺ III	8.0 (7, 9)	12 (16, 10, 10, 12)	1.0 (3, 0, 1, 0)	14 (14)	0.5 (0, 1)
pSV ori ⁻ III	25 (25)	31 (31, 28, 35, 32)	20 (19, 27, 22, 13)	60 (60)	18 (16, 20)
Salmon sperm	0.0 (0, 0)	0.0 (0, 0, 0, 0)	0.0 (0, 0, 0, 0)	0.0 (0, 0)	0.0 (0, 0)

SV40 virion form I DNA was extracted from SV40-infected monkey kidney cells (CV-1) by the technique of Hirt¹⁸ and isolated by CsCl-EtBr equilibrium centrifugation¹⁹. The DNA was further purified by deproteinization with redistilled phenol/CHCl₃/isoamyl alcohol, precipitated in 2 volumes 95% ethanol, redissolved in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and stored at 4 °C before use. pSV ori⁺ and pSV ori⁻ plasmid DNAs were isolated from *Escherichia coli* χ 1776 by the clear lysate technique²⁰ and purified as described above. Form III derivatives were prepared by digestion of form I with *Xho*I (which makes a single cut within pMK16); DNA was deproteinized and reisolated after ethanol precipitation. HS74 were utilized at the eighth passage. Culture medium was a 1:1 mixture of Dulbecco's modified Eagles medium (DMEM) and Ham's F-10 (MABioproducts) supplemented with 15% fetal calf serum ('complete media'), unless otherwise noted. Cells were grown in a 5% CO₂ atmosphere at 37 °C. DNA transfections were performed essentially as described by Wigler *et al.*²¹; 20 h before transfection, cells were plated at 7.5×10^5 per 60 mm Petri dish. Cultures were refed 4 h before transfection with 4.5 ml of DMEM supplemented with 5% calf serum. DNA-calcium phosphate precipitates were prepared such that each dish received 0.5 ml containing 0.5 μ g SV40 genome equivalents and 20 μ g high molecular weight salmon sperm DNA (as carrier). Cells were exposed to DNA for 4 h, then refed with complete medium. Cultures were incubated for 3 weeks and scored for transformed foci (*in situ* selection). In the subculture protocol, cultures were trypsinized 3 and 7 days post-transfection; one-half of each dish was reseeded into a 100 mm Petri dish and scored 3 weeks later for focus formation; the other half was suspended in 0.35% agarose⁵, and scored after 3 weeks for macroscopic colonies (0.1 mm in diameter).

* Data presented as average number of foci or colonies per plate, with individual points in parentheses.

Table 2 Transformation of HF by form III DNAs

DNA derivative	<i>In situ</i> foci	3 Day subcultivation		7 Day subcultivation	
		Foci	Colonies in agarose	Foci	Colonies in agarose
SV40 virion III	2.5 (1, 4)	3.3 (3, 5, 2, 3)	0.3 (0, 1, 0)	7.0 (1, 13)	0.0 (0, 0)
SV40 virion <i>Eco/Bam</i>	1.5 (1, 2)	2.5 (2, 3, 2, 3)	0.5 (1, 0, 1, 0)	0.0 (0, 0)	0.0 (0, 0)
SV ori ⁺ III	4.5 (1, 8)	6.7 (4, 7, 9)	2.0 (4, 0, 2, 2)	9.0 (9)	1.5 (0, 3)
SV ori ⁻ III	9.5 (8, 11)	8.3 (10, 5, 8, 10)	4.3 (5, 6, 3, 3)	25 (25)	20 (19, 22)

SV40 virion form I DNA was extracted from SV40-infected monkey kidney cells as described in Table 1 legend. Virion form III DNA was generated by digestion of form I DNA with *Bam*HI. The subgenomic *Eco/Bam* fragment was isolated by simultaneous digestion of virion form I DNA with *Eco*RI and *Bam*HI; the DNA was then electrophoresed on a 1% preparative agarose gel in Tris-borate buffer at 25 V for 18 h. DNA was visualized under UV light by ethidium bromide staining; and the 4,670 base pair fragment was cut out from the gel and reisolated by electroelution²². pSV ori⁻ and pSV ori⁺ plasmids were isolated as described in Table 1 legend. SV ori⁻ and SV ori⁺ form III DNAs (viral sequences from which plasmid sequences were removed) were generated by digestion of form I plasmid DNA with *Bam*HI, which separates SV40 from pMK16 sequences; viral DNA was isolated by agarose electrophoresis and electroelution as above. Cell culture, DNA transfections and all transformation assays were performed as described in Table 1 legend. Restriction enzyme digests were performed according to suppliers' (New England Biolabs and Bethesda Research Labs) specifications.

environment (such as methylation) appears likely as well because SV ori⁺ form III transforms with higher efficiency than viral DNA isolated from permissive monkey cells. Note, however, that the contribution of bacterial modification seems to be transient, and almost completely disappears after 7 days (see Tables 1, 2). Second, we tested whether secondary infection was responsible for the reduced transformation efficiency of viral DNA by using a viral DNA molecule which had been deleted of 750 base pairs in the late region, precluding formation of viral particles^{15,16}. No improvement was observed (compare lines 1 and 2 in Table 2).

These experiments demonstrate that the efficiency of transformation of semipermissive human fibroblasts can be markedly increased by using a viral DNA which is defective at the origin of DNA replication. Transformation with SV40 ori⁻ mutants resulted in an increased number of transformants as observed by both direct assay (for example, *in situ*) and subculture assays for anchorage independence. Furthermore, foci and agarose colonies generated with pSV ori⁻ DNA were larger than those generated by SV40 derivatives containing a functional viral origin of replication (see Fig. 1). The transformation frequency can be further increased by using linearized pSV ori⁻ DNA which gives markedly better results than conventional viral DNA I. The frequency of transformation of HF by pSV ori⁻ DNA increases as a function of the amount of DNA used in transfection. However, this increase is non-linear, and approximating threefold over the range 1–10 µg per culture. Increasing the amount of SV40 virion DNA in the same manner does not substantially influence the transformation frequency (data not shown). We are now investigating whether HF transformed by SV ori⁻ DNA are more likely to become permanently established¹⁷. Preliminary Southern blot analysis of the Hirt (low molecular weight DNA) supernatants of transformants generated by various SV40 derivatives has failed to detect production of free SV40 viral DNA in SV ori⁻-derived transformants, as predicted (D. Neufeld and H. Ozer, unpublished results).

In conclusion, we believe that the origin-defective recombinant SV40 molecule is the vector of choice for SV40 transformation of human fibroblasts. Its enhanced transformation efficiency (relative to wild-type SV40) is probably due to its inability to excise and replicate in transformed semipermissive cells. This property should increase the stability of the transformed phenotype. Furthermore, it precludes the possibility of cell death mediated by uncontrolled viral DNA synthesis or production of virus particles. We observe that SV ori⁻ DNA linearized in its plasmid sequence by digestion with the restriction enzyme *Xho*I routinely gives the highest transformation frequencies (~1 in 10⁵ or greater can be obtained on transfection of early passage cells). Transformants of human fibroblasts generated by this vector may prove particularly useful in the establishment of human cell lines for various purposes.

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Enhanced metastasis of tumours induced by a SV40 small T deletion mutant

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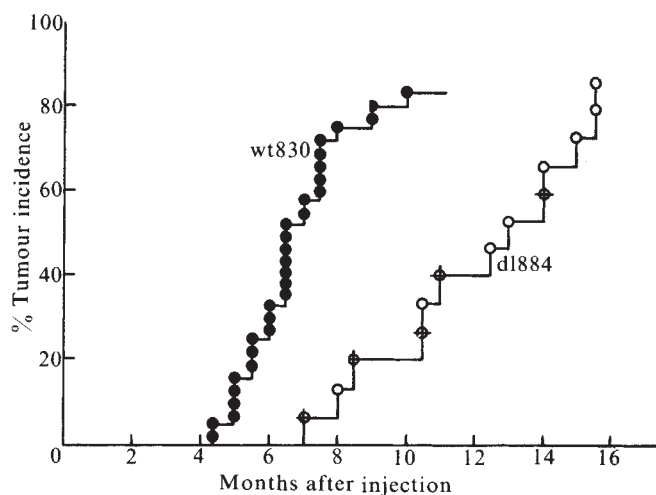
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The lethality of tumours is determined largely by their ability to metastasize to distant sites. Once metastasis has occurred, eradication of the tumour cells from the body becomes difficult or impossible. Although the steps involved in tumour metastasis have been well documented (for review see refs 1–3), the cellular parameters that influence the tendency of tumours to metastasize have been more difficult to define^{4–7}, and the underlying genetic determinants of metastatic potential are not known. We are studying the metastasis of tumours induced in hamsters by simian virus 40 (SV40). We show here that tumours induced by a viral mutant (d1884) in the small T antigen gene tend to metastasize, whereas tumours induced by wild-type SV40 rarely do so. Thus, we have a well defined system in which the underlying genetic differences in the transforming viruses are known and in which we may be able to correlate these differences with cellular parameters that determine tumour metastasis.

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Fig. 1 Time course of tumour induction by wild-type SV40 (wt830) and the small T deletion mutant (d1884). Newborn (<24 h of age) Golden Syrian hamsters (strain LVG, Charles River) were injected subcutaneously between the shoulder blades with $\sim 1 \times 10^8$ plaque-forming units (PFU) of either viral strain. (The small T deletion mutant d1884, constructed from the parental strain wt830 by Shenk *et al.*¹⁶, eliminates 253 base pairs¹⁷ in the 0.54–0.59 map unit segment of the early region of SV40. Thus, the coding sequence for the C-terminal 63 amino acids of the 174 amino acid small T protein are deleted. The coding sequences for the large T protein remain intact.) Animals were palpated for tumours at least once per week and the age of the animal at the time the tumour was first observed was recorded. The 'latency period', or the time required for the tumour to become observable, is plotted on the abscissa. Thirty animals were in the group injected with wild-type virus and 15 were in the group injected with the mutant virus. Animals displaying metastases are indicated by the crossed symbols.



the subcutaneous injection site. In some cases tumour foci were found only on internal organs or in the abdominal cavity, whereas in others both subcutaneous tumours and distant tumour foci were present (see Table 1). In all cases histological examination showed the neoplastic cells in the tumours and organs to be fibrosarcomas.

Of 36 animals injected with wt830, 30 animals developed tumours. The tissues of 12 animals with wild type-induced tumours were examined macroscopically and histologically and no abdominal tumours or metastases were observed.

To prove that the subcutaneous tumours and distant tumour foci actually arose from d1884-transformed cells, we have

We first compared the ability of wild-type SV40 (wt830) and the small T deletion mutant (d1884) to induce tumours following subcutaneous injection in newborn hamsters. The d1884 mutant is deficient in transformation of rodent cells in culture in some conditions^{8–10} and it was shown to cause tumours in animals at reduced frequency^{11,12}. We found that d1884 induces tumours at a similar frequency to wild-type SV40 but that these tumours appear after a longer latency period (Fig. 1)—a median latency period of 12.5 months, compared with 6.5 months for wild-type virus. The most striking finding, however, was that some of the animals injected with the d1884 virus (indicated by crosses in Fig. 1) developed tumour foci at sites distant from

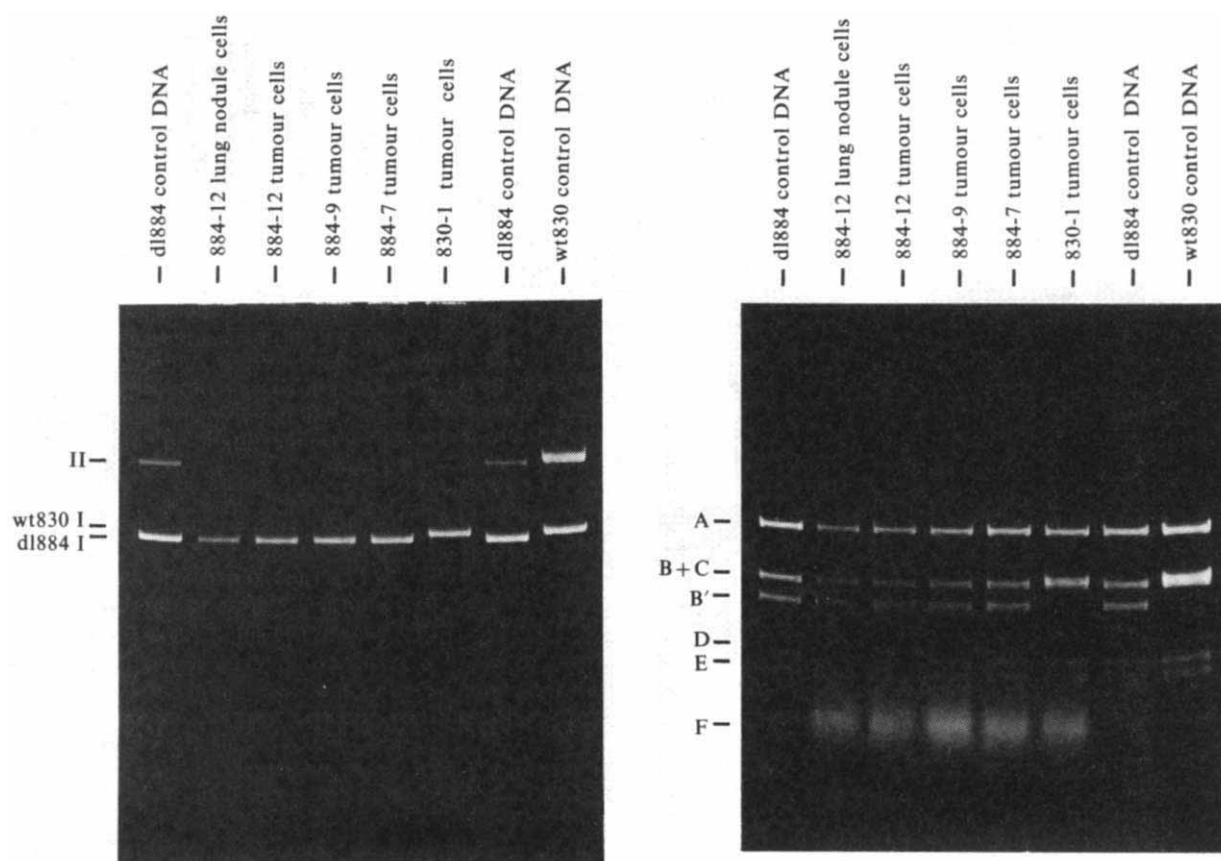


Fig. 2 Agarose gel electrophoresis of DNA from virus rescued from SV40-induced tumours. Tumours were removed from the animals and cell lines established by disaggregation of tumour pieces with collagenase and culture in minimal essential Eagle's medium with Earles salts, 5% fetal calf serum and 5% calf serum. Virus was rescued from these cell lines by culturing them together with permissive CV-1 cells (African green monkey kidney line CV-1 clone TC 7; ref. 18). Virus was collected after 14 days and used to infect more CV-1 cells. After 3 days the viral DNA was extracted from these cells by the method of Hirt¹⁹. The extracted DNA was electrophoresed on 0.8% agarose gels or cleaved with *Hind*III (New England Biolabs) and then electrophoresed. The direction of migration is from top to bottom in the photograph. Left panel, uncleaved viral DNA; right panel, viral DNA cleaved with *Hind*III. The numbers above designate individual animals. Roman numerals to the side refer to SV40 form I and II molecules. Letters to the side refer to the *Hind*III restriction fragments. The mutant d1884 deletion is within fragment B.

Table 1 Characteristics of hamsters exhibiting metastases

Animal	Site of principle tumour	Time at which hamster died or was killed	Location of metastases
884-1	None	Died at 8.5 months	Lung, spleen
884-2	Abdominal	Died at 7 months	Peritoneum, lung
884-5	None	Died at 10.5 months	Liver, pancreas, colon and lymph node
884-6	Abdominal	Died at 11 months	Spleen, liver and intestine
884-9	Subcutaneous, abdominal	Killed at 14 months (14 days after subcutaneous tumour was observed)	Peritoneum, diaphragm, liver
884-12	Subcutaneous	Killed at 11 months (45 days after subcutaneous tumour was observed)	Pleural surface

Animals were necropsied as soon as possible after death or immediately after killing. Gross observations were made and tissues prepared for histology.

characterized virus rescued from cultured tumour cells by co-cultivation with permissive monkey cells (CV-1 line). Rescued virus was used to infect CV-1 cells and viral DNA was prepared from a Hirt supernatant fraction. Cleaved (*Hind*III) and uncleaved viral DNA was electrophoresed on 1% agarose gels, stained with ethidium bromide and photographed (Fig. 2). Viral DNA rescued from d1884-induced tumour cells co-electrophoreses with d1884 viral DNA; viral DNA rescued from wt830-induced tumour cells co-electrophoreses with wt830 viral DNA. Note that d1884 viral DNA was rescued from cells recovered from a metastatic lung nodule as well as from the subcutaneous primary tumour.

The distant tumour foci that we observed arose either as metastases from a primary tumour or by independent virus-mediated transforming events. These two possibilities may be distinguished by comparing the viral genome integration pat-

terns in cells from the primary tumours with those in cells from the distant tumour foci. If the distant tumour foci arose by independent transforming events, the viral genome integration patterns would be likely to differ from those of the primary tumour, because SV40 viral DNA seems to integrate at random^{13,14}. If these distant tumour foci are metastases from the primary tumour, their viral integration patterns should be the same.

We analysed the viral genome integration patterns from primary tumours and metastases. Cellular DNA was extracted from cultured tumour cells, treated with restriction endonucleases, electrophoresed on agarose gels, transferred to nitrocellulose sheets¹³ and hybridized with the ³²P-labelled nick-translated SV40 DNA probe¹⁴. Analysis of the integration patterns in cultured cells from several of the primary subcutaneous tumours, using restriction enzymes that cut within the SV40

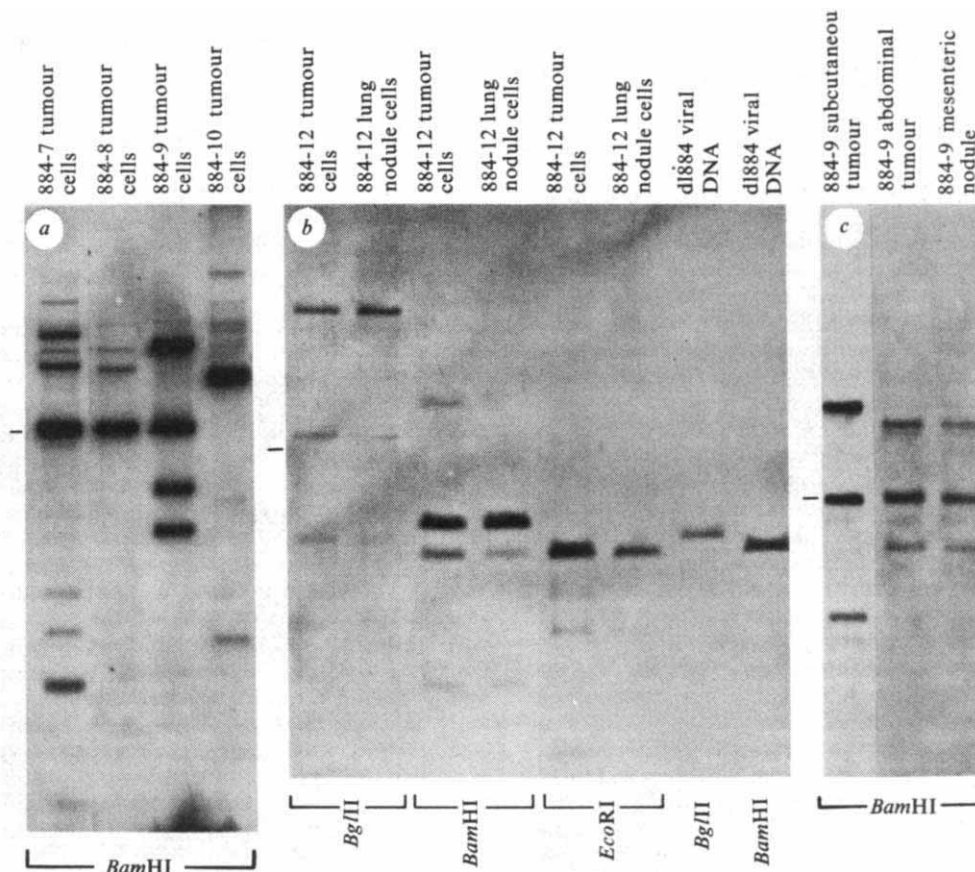


Fig. 3 Hybridization of restriction endonuclease-cleaved cellular DNA from small T deletion mutant-induced tumours and metastases with the SV40 probe. Cellular DNA was extracted from cultured tumour cells by the method of Botchan *et al.*²⁰ or from frozen tumour pieces as described by Blin and Stafford²¹. DNA was then cleaved with *Bam*HI, *Bgl*II or *Eco*RI (New England Biolabs) and electrophoresed on a 0.8% agarose gel. DNA was transferred to nitrocellulose (as described by Botchan *et al.*¹³) and hybridized with ³²P-labelled nick-translated SV40 DNA (~1 × 10⁸ c.p.m. per µg) according to the method of Wahl *et al.*¹⁴. The position of the unit length linear SV40 band is indicated by the dashes in the figure. *a*, Cellular DNA was prepared from cultured cells derived from primary tumours of four different animals. *b*, Cellular DNA was prepared from cultured cells derived from a primary tumour and a lung nodule from the same animal. (The *Bgl*II enzyme preparation contains a nicking activity that converts SV40 form I DNA to form II.) *c*, Cellular DNA was extracted from frozen tumour pieces of three separate tumours present in a single animal.

Table 2 Metastasis of re-injected tumour cells

Tumour cells	No. of cells inoculated	Tumour incidence	Tumour latency [median (range), in days]	Metastasis incidence
884-12	3×10^4	4/4	25(25-30)	4/4
884-12	3×10^3	5/5	30(25-37)	5/5
830-1	3×10^4	5/5	30(25-92)	1/5
830-1	3×10^3	3/5	81(49-91)	0/5

Tumour cells were passed in culture, then trypsinized and injected subcutaneously into 7-week old hamsters. Animals were killed and examined for metastases ~42 days after the subcutaneous tumours were first observed.

genome either once or not at all, indicated that the integration patterns are relatively simple (only a few integration sites) and that there is a small amount of free viral DNA. Furthermore, we found a unique pattern in each of the cell lines derived from subcutaneous tumours in different animals (Fig. 3a). Most importantly, we found that the integration pattern in cells from a primary tumour and lung nodules from the same animal are identical (Fig. 3b). Thus, we conclude that the cells in the lung tumour nodules are actually derived by metastasis from the subcutaneous primary tumour. However, not all of the distant tumour foci seem to be derived as metastases from a subcutaneous tumour. For example, one animal had a subcutaneous tumour and an abdominal tumour exhibiting distinct viral integration patterns (Fig. 3c). Additional small abdominal tumour foci had patterns identical to the major abdominal tumour (Fig. 3c). Thus, either the abdominal tumours were derived as metastases from an undetected subpopulation of cells in the subcutaneous tumour or they arose from an independent transforming event perhaps occurring in the abdomen. We never observed abdominal tumours following subcutaneous injection of the wild-type virus. If the development of the abdominal tumours is unrelated to metastasis, it could be due to a change in the cell specificity of the virus particle itself.

What causes the difference in metastasis of tumours induced by wild-type SV40 and the small T deletion mutant? We believe that the observed metastasis is not a result of the increased latency period exhibited by the small T deletion mutant. Metastatic tumours arose after latency periods similar to non-metastatic tumours observed in many of the animals injected with the wild-type virus. Furthermore, it is unlikely that the metastasis is due to a prolonged presence of the primary tumour in the animal. Deichman and Kluchareva¹⁵ showed that metastasis of wild type-induced tumours sometimes occurred when tumours were allowed to develop for more than 60 days after they had first appeared; in our experiments, animals were usually killed at much earlier times after the tumours first appeared because later on the tumours become quite large and the animals appeared sickly. Instead, the tumour metastasis seems to be related to properties of the tumour cells themselves. We have determined that cells derived from metastatic tumours and passed in culture retain their ability to metastasize on reinjection in adult animals; cells from wild type-induced tumours metastasize less readily in these conditions (Table 2). Topp *et al.*¹² compared cells derived from wild type- and small T mutant-induced tumours for certain transformed properties (the ability to grow suspended in methylcellulose, loss of actin-containing cable networks and synthesis of plasminogen activator). Although no differences between the wild type- and mutant-induced tumour cells were observed, metastasis was not monitored in their studies, and it is unclear whether any of the cell lines analysed were derived from metastatic tumours.

Further analysis of the properties of our tumour-derived cell lines may reveal characteristics which explain their difference in metastatic potential. Once these characteristics have been identified, we will be able to investigate how they are determined by the genetic make-up of the transforming viruses.

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Activation of non-expressed bovine papilloma virus genomes by tumour promoters

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Naturally occurring cancer seems to be a multiple-hit phenomenon resulting from the interaction of several factors. Numerous environmental carcinogens on one hand and the widespread tumour viruses on the other, are capable of either transforming cells *in vitro* or inducing tumours *in vivo*. A few years ago, based on epidemiological evidence, a possible interaction between bovine papilloma viruses and an environmental carcinogen in bracken fern was noted in conjunction with alimentary cancer in cattle¹. We report here that tumour-promoting agents which by themselves are unable to transform cells, are capable of inducing the transcription of bovine papilloma virus type 1 (BPV-1) in mouse embryo fibroblast (MEF) tissue cultures, where the viral genomes reside in a non-expressed episomal state after infection. In such cells, one brief treatment with the tumour promoter results in the transcription of the same viral mRNA species present in BPV-1-induced tumours and in BPV-1-transformed cells. Furthermore, viral DNA is replicated and the cells acquire the transformed phenotype. Once activated, these properties remain stable. This interaction between tumour promoters and latent inactive tumour virus genomes leads, in appropriate cell systems, to the activation of particular viral genes and to the transformed phenotype of the host cells.

BPV can induce fibroblastic tumours in animals²⁻⁴ and is able to transform tissue culture cells^{5,6}. Unlike most other tumour viruses, however, BPV achieves transformation as an unintegrated episome⁷⁻¹². A further peculiarity of BPV is the lack of a permissive tissue culture system. Virion production occurs exclusively in the differentiated periphery of the virus-induced warts¹³. Hence, as a rule, infection of tissue culture cells leads, if at all, to only a partial expression of the viral genome¹⁴ with ensuing transformation. Not all tissue isolates from one species are transformable; only 2 of 11 fetal bovine skin cultures could be transformed with BPV¹⁵. Similarly, not all mouse-derived

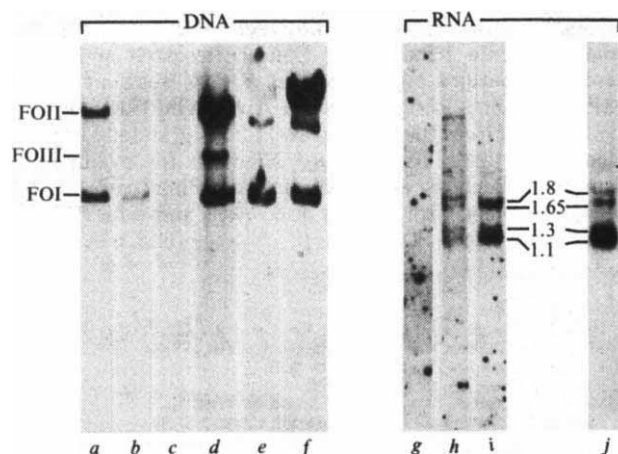


Fig. 1 Effect of TPA on BPV-1-infected MEF cells. MEF cells (DBA strain, passage 5) were infected with a BPV-1 virus pool (0.5 ml per 10^7 cells) which had been prepared as follows: a bovine wart was minced in an Omnimixer at 4 °C for 10 min (10% w/v in phosphate-buffered saline). After pelleting at 20,000g for 20 min, the virus-containing supernatant was sterilized by passage through a Millipore filter and used to infect MEF cells. The viral DNA showed a typical BPV-1 restriction enzyme cleavage pattern⁷. The infected cells were passaged once a week at a 1:5 split ratio in Eagle's basal medium supplemented with 10% fetal bovine serum. DNA and RNA were prepared 4 days after plating when the cells had reached confluence, and were analysed by gel electrophoresis either in 1% or 1.4% agarose as described elsewhere²². After phenol extraction, the RNA was selectively precipitated with 2 M LiCl and traces of DNA were removed by treatment with DNase I (grade I) as detailed elsewhere¹⁴. Transfer to nitrocellulose filters, hybridization with a nick-translated ³²P-labelled BPV-1 DNA probe (cloned in pBR322 and provided by P. Howley), and autoradiography were as described elsewhere²². In each track of the gels 5 µg of DNA and 20 µg of RNA were assayed. *a-f*, DNA from BPV-1-infected MEF cells. *a-c*, 1, 2 and 3 passages, respectively, after infection; *d*, same as *a*, but exposed for 4 days to 10^{-7} M TPA; *e*, same as *d*, but in the first passage after removal of TPA; *f*, same as *d*, but in the fifth passage after removal of TPA. *g-i*, RNA from the same cultures as above. *g*, MEF cells 1 passage after infection; *h*, 1 passage after infection and exposure for 4 days to 10^{-7} M TPA; *i*, same as *h*, but in the second passage after removal of TPA; *j*, RNA from BPV-1-transformed hamster embryo fibroblast cells in the 16th passage after infection. The values on the right indicate the sizes of the BPV-1 RNA sequences¹⁴ in kb.

fibroblast tissue cultures could be transformed, for example, NIH 3T3 and C 127 responded to BPV-1 infection with transformation⁸, while repeated efforts in our laboratory to transform fibroblasts from the DBA strain were unsuccessful.

Following passaging of the BPV-1-infected phenotypically normal cells, the viral DNA is eventually lost because of its failure to replicate. This is shown in Fig. 1*a-c*, where the method of Southern¹⁶ was used to reveal BPV-1 DNA after infection of MEF cells with BPV-1 virions. One passage after infection, superhelical form I (FOI) and relaxed FOII BPV-1 DNA were detected in the cells using a ³²P-labelled, nick-translated BPV-1 DNA probe. After the second passage, most of the viral DNA had disappeared (Fig. 1*b*), and after a third passage no viral DNA was detectable (Fig. 1*c*). It has been shown that 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has a stimulatory effect

on Epstein-Barr virus¹⁷ and episomal papovavirus genomes¹⁸, and this is confirmed in Fig. 1*d*. A parallel culture to that shown in Fig. 1*a* was subjected to TPA treatment and, when examined 4 days later displayed considerably more BPV-1 DNA, particularly FOII and some unit-length FOIII. This effect was observed as early as 18 h after treatment with TPA. Even when administered to cells in the third passage after infection, TPA amplified the minute amounts of persisting non-detectable BPV-1 DNA (see Fig. 1*c*) to the extent that a clearly visible signal was obtained by the Southern method (data not shown).

Furthermore, the BPV-1 DNA continues to replicate in the absence of TPA. Figure 1*e, f* show the viral DNA content at one and five passages after TPA removal. In addition to FOI and FOII, multimeric BPV-1 DNA structures are present. These can be converted to linear FOIII after cleavage with single-cut restriction endonucleases (data not shown).

The effect of TPA on viral transcription is shown in denaturing gels (Fig. 1*g-j*)¹⁹. The total RNA was extracted from the same cultures used to analyse DNA. Although untreated MEF cultures contain BPV-1 DNA one passage after infection (Fig. 1*a*), no viral RNA sequences could be detected (Fig. 1*g*). However, in a parallel culture, 4 days after TPA treatment (Fig. 1*h*; see Fig. 1*d* for the DNA content), several species of BPV-1 RNA were found. The same RNA species are also found in the polyadenylated RNA of BPV-1-induced hamster tumours and BPV-1-transformed hamster cells¹⁴ (Fig. 1*j*). That these bands represent true RNA sequences is shown by their absence after RNase or alkali treatment (data not shown). Viral transcription persisted during subsequent passages in the absence of TPA (Fig. 1*i*). In agreement with our previous findings, the 2-kilobase (kb) mRNA which probably encodes the viral capsid protein and which is present exclusively in the permissive periphery of the warts¹⁴, was absent. The faint larger bands were observed only occasionally and may represent mRNA precursors.

Together with the induction of viral transcripts, TPA also induced morphological alterations of the cells. We observed a saturation density 25% higher than the control (uninfected TPA-treated) or infected (untreated) cultures 6 days after temporary TPA treatment (18 h). Furthermore, several passages after TPA treatment, the BPV-1-infected cultures had acquired the ability to form colonies in medium having low serum content. This transformed phenotype was not evident in control cells.

We obtained similar results with a newly developed compound, 12-*O*-retinoylphorbol-13-acetate (RPA)²⁰, which combines the anti-promoting vitamin A with a phorbol ester tumour promoter. RPA exerts a mitogenic effect on the skin of NMRI mice similar to that induced by TPA. When applied to BPV-1-infected MEF tissue culture cells, RPA activated the latent non-expressed viral genomes in a manner similar to TPA (Table 1).

However, not all cell lines provide a suitable environment for the above interactions (Table 1). While primate cells did not respond at all, a temporary activation of BPV-1 DNA replication in the absence of transcription was achieved by TPA

Table 1 Effect of TPA and RPA on BPV-1 episomes

Cell type	Designation	DNA replication	Transcription	Transformation
Monkey kidney	CV-1	-	-	-
Human foreskin fibroblasts	HFF	-	-	-
Human embryonic lung	HEL	-	-	-
Bovine kidney	NBL	Transient	-	-
Bovine fetal thyroid	BFT (ref. 21)	+	Transient	Transient
Rat embryo fibroblast	REF	Transient	-	-
Mouse embryo fibroblast	MEF*	+	+	+

DNA replication and transcription were determined by the method of Southern¹⁶ 4 days after addition of 10^{-7} M TPA or RPA. Both compounds were applied separately to all cell types. Transformation reduced serum requirement and increased saturation density. -, No effect observed. 'Transient' denotes that the effect was abolished after two passages following TPA or RPA treatment. +, BPV-1 DNA replicates in the absence of tumour promoters.

* DBA mouse strain, fifth passage; cells show a limited *in vitro* lifetime unless infected and treated with either TPA or RPA.

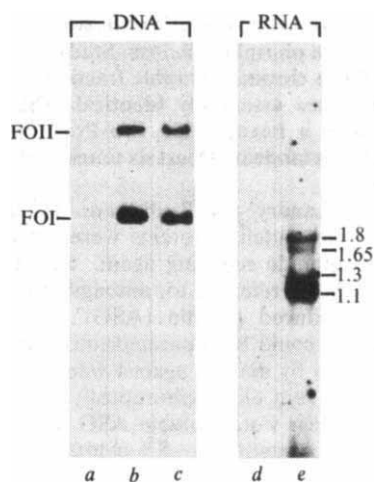


Fig. 2 Effect of RPA on BPV-1-infected BFT cells. BFT cells were infected with BPV-1 and passaged as described in Fig. 1 legend. Preparation and characterization of BPV-1-specific DNA and RNA sequences are also described in Fig. 1 legend. *a-c*, DNA from BFT cells. *a*, Uninfected; *b*, four passages after infection; *c*, as in *b*, but treated for 3 days with RPA (10^{-7} M); *d*, RNA from the same culture as in *b*; *e*, RNA from the same culture as in *c*. The values indicate the sizes of the BPV-1 RNA sequences in kb.

treatment of NBL cells. BFT cells, also of bovine origin, which are free of endogenous BPV-1 genomes (Fig. 2*a*), when infected with BPV-1 were capable of supporting the continuous replication of episomal BPV-1 DNA (Fig. 2*b*). However, no viral transcripts were observed (Fig. 2*d*) and a normal phenotype was retained. After exposure to RPA (or TPA, data not shown), no additional effect on the BPV-1 DNA content was observed (Fig. 2*c*), but transcription of the BPV-1 genomes was initiated (Fig. 2*e*) and the cells acquired a transformed phenotype (Fig. 3). However, these effects were transient, as transcription of the BPV-1 episomes was abolished after two further passages in the absence of promoter, despite the continuing replication of BPV-1 DNA. It is interesting that the relative amounts of BPV-1 transcripts obtained after RPA (and TPA) treatment corresponded initially to, or even exceeded those observed in BPV-1-transformed hamster embryo fibroblast (HEF) cells (Fig. 1*j*) which contain comparable numbers of episomal DNA, that is, ~50–100 per cell. Treatment with the tumour promoters failed to enhance the relative amount of BPV-1-specific transcripts in the latter case (not shown).

Thus, it seems that the promoter-treated BFT and MEF cells may constitute an appropriate host system for amplification and expression of foreign genes which have been inserted into a BPV-1 vector. Furthermore, the experimental system described here may be used for rapid screening of putative tumour-promoting agents, thereby reducing the need for time-consuming studies in laboratory animals.

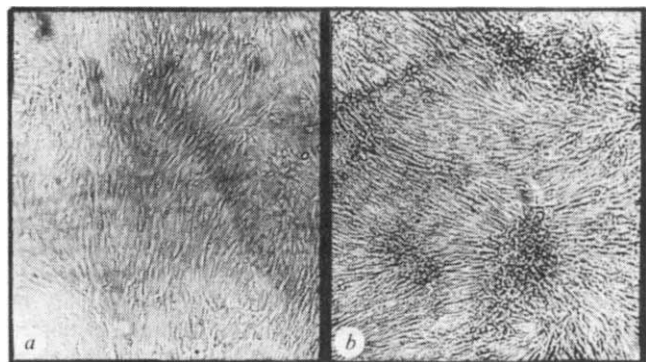


Fig. 3 Effect of RPA on BFT cells. BPV-1-infected cells (0.1 ml BPV-1 per 2×10^6 cells) were passaged (1:5 split ratio) 4 days after infection: *a*, 7 days after passaging in medium without RPA; *b*, parallel culture, but maintained for 7 days in medium containing 10^{-7} M RPA.

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Electron microscopy of hepatitis B core antigen synthesized in *E. coli*

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DNA fragments from hepatitis B virus (HBV) have been inserted into plasmid vectors and cloned in *Escherichia coli*¹, and some of these bacterial clones have been reported to synthesize hepatitis B core antigen (HBcAg)^{1,2}. Material from a derivative of one HBcAg-synthesizing clone is being evaluated in our laboratory and elsewhere^{3,4} as a serological reagent for use in various assays, including immune electron microscopy, to detect anti-HBcAg antibody. It has been shown by gel exclusion chromatography that the bacterial HBcAg is in an aggregated form⁵. We now report that the *E. coli* HBcAg preparation contains small round particles similar in appearance to the viral cores previously seen in material obtained from HBV-infected humans^{6–7} and chimpanzees⁸.

Extracts of *E. coli* harbouring the recombinant plasmid pRI-11 (ref. 3) and of human liver⁹ containing HBcAg were examined by immune electron microscopy (Fig. 1). The morphology of particles precipitated by anti-HBcAg antibody from the bacterial HBcAg preparation was similar to that of viral cores seen in extracts from HBV-infected liver. The average diameter of the particles from *E. coli* was 27.3 nm (range 24–31 nm, *n* = 100); that of particles in the liver extract was 28.1 nm (range 24–32 nm, *n* = 100).

Preliminary density gradient studies have shown that the *E. coli*- and liver-derived HBcAg give bands at 1.35–1.36 g ml⁻¹ in caesium chloride, indicating that they contain nucleic acid; this was confirmed by the UV absorption spectra of both antigens (P. Wingfield and K. Murray, personal communication).

Further immune electron microscopy tests have confirmed that the particles seen in the *E. coli* preparation possess the characteristic antigenic properties of HBcAg. Results of these studies and of other confirmatory tests will be reported elsewhere.

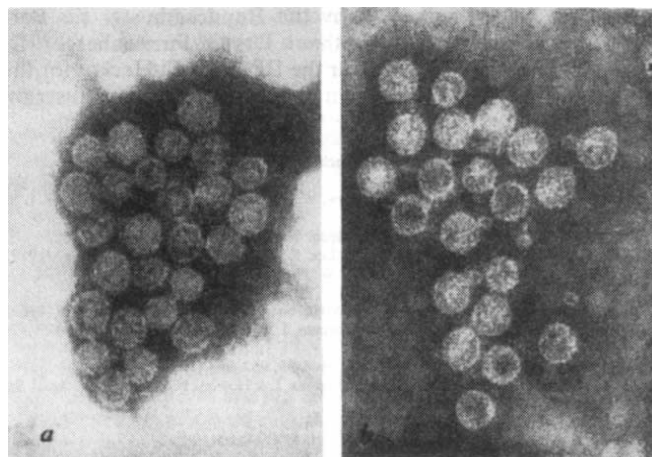


Fig. 1 Immune electron microscopy of *a*, *E. coli*-derived and *b*, human liver-derived HBcAg ($\times 189,000$). Each antigen (0.1 ml) was mixed with an equal volume of human anti-HBcAg IgG ($2\text{--}400\ \mu\text{g ml}^{-1}$), incubated at 20°C for 1 h, then diluted to 2.5 ml with phosphate-buffered saline and centrifuged at $48,000g$ for 1 h. The resulting pellet was resuspended in the minimum amount of distilled water ($<0.1\ \text{ml}$) and mixed with an equal volume of negative stain (3% phosphotungstic acid, pH 6.3). A drop of the mixture was placed on a Formvar-carbon-coated grid which was then blotted dry with filter paper. The specimens were examined in an AEI 801 or a Jeol 100 CX electron microscope with magnifications calibrated using crystalline catalase¹⁰.

Electron microscopy has thus revealed the presence of small round particles in extracts of *E. coli* that carry a hybrid plasmid directing the synthesis of HBcAg. The morphological and antigenic properties of these bacterial HBcAg particles are comparable with those of naturally occurring HBV cores.

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Tandem repeats in the N-terminal sequence of a proline-rich protein from corn endosperm

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Zeins, the prolamines of corn (*Zea mays*), are the most abundant storage protein fraction in corn endosperm. Like prolamines of other cereals, zeins are readily soluble in 60–70% alcohol solutions but are insoluble in aqueous buffer systems. The other major storage protein fraction in corn endosperm is glutelin, which is extractable with either dilute alkali or detergent. We have now extracted from corn endosperm a proline-

rich, zein-like protein fraction which we have purified by chromatography on phosphocellulose. Study of the N-terminal sequences of three chromatographic fractions for homologies shows that they are essentially identical. These N-terminal sequences include a hexapeptide Pro-Pro-Pro-Val-His-Leu, which is repeated in tandem at least six times, and probably eight times or more.

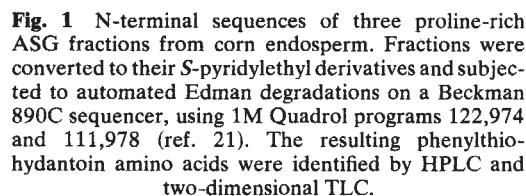
Moureaux and Landry¹ and Paulis *et al.*² independently discovered that some glutelin proteins were soluble in alcohol containing a disulphide reducing agent. This alcohol-soluble fraction of glutelin is referred to, amongst other names^{3–5} as alcohol-soluble reduced glutelin (ASG)⁶. Paulis and Wall⁷ showed that ASG could be separated into water-soluble and -insoluble fractions by dialysis against water. Water-insoluble ASG is similar to zein electrophoretically and in amino acid composition, whereas water-soluble ASG differs significantly. Water-soluble ASG constitutes ~5% of total protein in the corn endosperm (unpublished data).

We recently⁸ fractionated ASG by chromatography on phosphocellulose and found that three late-eluting fractions, fractions 4 (peak 4), 5 (peak 5) and 6 (the late-eluting tail of peak 5), contained proteins similar to either the water-soluble ASG⁷ or the reduced-soluble (RS) protein described by Wilson *et al.*⁹. The amino acid compositions of these ASG fractions are essentially identical, but differ significantly from zein⁸. They contain a large number of proline residues (~26 per 100 residues) and are exceeded in proline content only by certain proteins in wheat omega-gliadin¹⁰, barley C-hordein¹¹ and human saliva¹². SDS-polyacrylamide gel electrophoresis showed that all three fractions are homogeneous and have a molecular weight (M_r) of 27,500. In contrast, they show extensive charge heterogeneity (14–16 components, with 5 predominant ones) on isoelectric focusing (IEF). All three IEF profiles are essentially similar, however, the differences being primarily quantitative.

N-terminal amino acid sequence analysis revealed that these proline-rich ASG fractions have identical sequences for at least 49 residues (Fig. 1). This degree of sequence homology suggests that the polypeptides are products of homologous genes which arose by duplication from an ancestral gene. Major heterogeneity was evident at only two positions (1 and 9). Glycine and threonine were found in an approximately equimolar ratio at residue 1 of fraction 6; at residue 9, in addition to cysteine, asparagine was detected in fraction 4 (Cys/Asn ~ 1:1) and serine in fraction 5 (Cys/Ser ~ 1:2). The presence of serine and cysteine at the same position can be accounted for by a point mutation involving a single nucleotide substitution. In addition to positions 1 and 9, slight microheterogeneity was occasionally noted in the first nine positions, amounting to no more than 5–10% of the major determined residue. This may explain the heterogeneity of these fractions observed after IEF. There was no sequence heterogeneity apparent from residues 10–49, indicating possible evolutionary pressure for conservation of this sequence.

The N-terminal sequence of proline-rich ASG fractions 4–6 is extremely hydrophobic: 69% of the amino acids identified in the first 58 positions are proline, valine and leucine. In fact, proline accounts for 43% of the first 58 residues. On the basis of amino acid composition and estimated molecular weight⁸, it is expected that fractions 4–6 contain ~60 prolines out of a total of 225–230 residues, and the occurrence of 25 prolines in the first 58 positions indicates an uneven distribution of this amino acid. This is also true for histidine, where 9 (of 12–14 total expected) histidine residues occur in the first quarter of the molecule, making its N-terminus positively charged. Indeed, the occurrence of regularly spaced histidines in a region rich in proline, valine and leucine may be responsible for the solubility of fractions 4–6 in water, as charge repulsion between histidines could diminish hydrophobic interactions among nonpolar residues.

The most surprising observation from the data in Fig. 1 is that a hexapeptide, Pro-Pro-Pro-Val-His-Leu, is tandemly repeated at least six times, and probably a minimum of eight times, in



Obviously, tandem 6-residue repeats in proline-rich ASG fractions are colinear to 18-nucleotide-long DNA repeats. These DNA repeats may have arisen either by saltatory replication²⁴, by unequal crossing-over²⁵, or by one of the other mechanisms reviewed elsewhere²⁶. In fact, we postulate that repeats in proline-rich ASG represent an insertion into an ancestral prolamine gene from one of the repetitive sequence families of the maize genome, rather than from intracistronic tandem duplications involving an 18-nucleotide sequence. The isolation of mRNAs encoding proline-rich ASG polypeptides, the production of cDNA probes from these mRNAs, and hybridization of the probes with various components of maize DNA may elucidate the origin of these repeated sequences.

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Activation of transcription of a yeast gene in *E. coli* by an IS5 element

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pRG4 and pRG5 are two plasmids derived¹ from pBR322 which carry an integrated yeast DNA segment (2.5 kilobases) containing the *URA1* gene^{2,3} and differ only in the orientation of the insert. *Escherichia coli pyrD* cells transformed by pRG4 recover the ability to grow on minimal medium whereas pRG5-transformed *pyrD* cells do not³. The spontaneous integration of an insertion (IS) element into the cloned DNA segment corrects the absence of complementation in pRG5-transformed *pyrD* cells³. The new plasmid resulting from such an insertion event has been named pRG7 (Fig. 1). To determine the level at which the IS element affected *URA1* expression, we assayed the RNAs which hybridized to each strand of *URA1* DNA in various transformed *pyrD* strains, using as a probe single-stranded *URA1* DNA cloned in phage fd106⁴. The results showed that the switching on of yeast gene by the IS element occurred at the transcriptional level, strongly suggesting that this IS element can serve as a mobile promoter in *E. coli*. The IS element has been identified as an IS5.

To determine which strand served as a template for the transcription of *URA1*, we cloned the 2.5-kilobase (kb) fragment containing *URA1* into the *Hind*III site of the replicative form of phage fd106^{4,5}. Phages carrying the yeast DNA in both directions were obtained from infected KB35F⁺ cells⁴. DNA from each type of phage was used to assay the *URA1* mRNAs in the two isogenic yeast strains FL100 and *ppr1*-1⁶, a strain

constitutive for synthesis of dihydroorotate dehydrogenase. The regulatory mutation *ppr1*-1 affects transcription of the unlinked gene *URA1*, the level of *URA1* mRNA being about five times higher in the *ppr1*-1 strain than that in the wild-type strain². The results presented in Table 1 indicated that in the wild-type strain yeast RNA hybridized to both strands of the cloned yeast DNA. About 83% of these hybridized RNAs were complementary to one strand, arbitrarily named plus, 17% to the other strand named minus. In the *ppr1*-1 strain, the level of the RNA hybridizing to the plus strand was about five times higher than that found in the wild-type strain, whereas the amount of RNA hybridizing to the other remained almost unchanged, showing that the plus strand carried the sequence complementary to *URA1* mRNA. Hybridization to both strands of the 2.5-kb cloned DNA was still observed after subsequent purifications of the plus phage and thus could not be attributed to contamination of the plus strand by the minus strand. Rather, this suggested that the cloned fragment not only encompassed the entire *URA1* sequence but additionally contained the end of a second

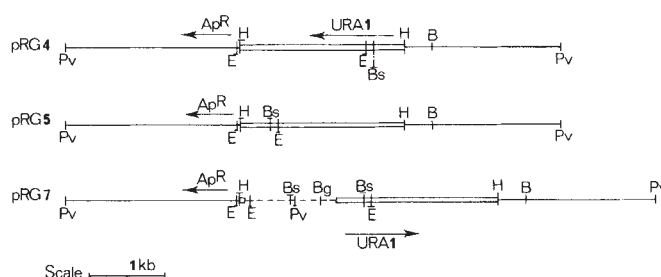


Fig. 1 Structure of plasmids pRG4, pRG5 and pRG7. The construction of these plasmids is described in refs 2 and 3. The DNAs are represented linearized after single cleavage at the *Pvu*II site of pBR322¹. pBR322 DNA sequences are represented by a continuous single line, yeast DNA sequences by an open box and the IS element DNA by a broken single line. The sites shown for restriction endonuclease cleavage are as follows: E, *Eco*RI; H, *Hind*II; B, *Bam*HI; Bs, *Bst*EII; Bg, *Bgl*II; Pv, *Pvu*II. The arrows indicate the direction of transcription of the ampicillin-resistance (*Ap*^R) gene¹ and of the *URA1* gene. kb, Kilobase.

Table 1 Transcription of the *URA1* region in yeast

Yeast strain	Amount of RNA hybridized to the plus strand of <i>URA1</i> DNA	Amount of RNA hybridized to the minus strand of <i>URA1</i> DNA
Expt 1 Wild-type FL100	1.2×10^{-5}	0.3×10^{-5}
<i>ppr1</i> -1	6.8×10^{-5}	0.4×10^{-5}
Expt 2 Wild-type FL100	3.3×10^{-5}	0.5×10^{-5}
<i>ppr1</i> -1	5.3×10^{-5}	0.9×10^{-5}

Yeast cells were grown in minimal medium (Difco yeast nitrogen base 6.7 g l^{-1} , 2% glucose) at 30 °C. Cells were labelled in exponential phase using ³H-adenine ($20 \text{ } \mu\text{Ci ml}^{-1}$) of specific activity 17 Ci mmol^{-1} . Yeast RNAs were extracted from 10-ml cultures^{15,16}. Conditions for hybridization of RNA to DNA fixed on nitrocellulose filters (Sartorius) are described in ref. 16. The nitrocellulose filters carrying probe DNA were prepared according to ref. 17. Specific hybridization of the cloned yeast DNA was measured in triplicate as follows: labelled RNA was incubated in a small glass vial with one filter loaded with $1 \text{ } \mu\text{g}$ fd-*ura1* plus strand DNA and one filter loaded with $1 \text{ } \mu\text{g}$ fd106 DNA. The strand which hybridized the most RNA was arbitrarily named the plus strand. To evaluate hybridization to each strand of the yeast DNA, c.p.m. retained on a fd DNA filter were subtracted from c.p.m. on a fd-*ura1* DNA filter. The amount of hybridization was expressed as the fraction of radioactivity specifically retained versus the input. Conditions were such that the input of radioactivity per unit cell mass was the same for both strains. Expts 1 and 2 are two completely independent experiments.

gene in the opposite strand. The orientation of the *URA1* region in each type of phage was determined by *Eco*RI restriction analysis of the corresponding replicative forms (not shown). The polarity of the virus strand being known⁴ and the template strand for *URA1* transcription determined to be the plus strand, the direction of transcription of *URA1* could be deduced to proceed from the internal *Eco*RI site towards the distal *Hind*III site (see Fig. 1).

We then assayed the RNAs which hybridized to each separated strand of the cloned yeast DNA in the *E. coli pyrD* strains transformed by pRG4, pRG5 or pRG7 (Table 2). In the pRG5-transformed *pyrD* strain, no dihydroorotate dehydrogenase activity was found, in agreement with our previous results³; no hybridization of RNA to the plus strand could be detected in typical pulse-label experiments, indicating that transcription of *URA1* did not occur in this strain. One could propose two explanations for this absence of transcription. The first would be that there is a signal somewhere in the 5' end of the cloned gene that is recognized as a stop by the *E. coli* RNA polymerase and therefore impedes the transcription of *URA1*, an hypothesis which can be discarded because *URA1* is transcribed when cloned in the opposite direction. The second explanation would be that no yeast sequence in this cloned segment is recognized as a promoter by the *E. coli* RNA polymerase; the transcription of *URA1* would therefore depend on the existence of a pBR322 promoter and would only

occur by a readthrough mechanism. In support of this hypothesis, Stüber and Bujard⁷ have found that the ampicillin-resistance gene of pBR322 could be transcribed from two promoters, one of which is located near the *Hind*III site and can serve to initiate the transcription of DNA segments inserted into this site. Notably, the direction of transcription of *URA1* in pRG4 coincides with that of the ampicillin-resistance operon. No pBR322 promoter can be used to transcribe *URA1* in pRG5, as the promoter of the tetracycline-resistance operon is inactivated by *Hind*III cleavage⁷. However, when *URA1* is integrated into the *Bam*HI site, which is located inside the structural gene for tetracycline resistance, again only one orientation of the cloned fragment allows expression of the *URA1* gene³. In this case, the direction of transcription of *URA1* coincides with that of the tetracycline-resistance operon. These results strongly support the hypothesis that *URA1* requires a bacterial promoter for its expression in *E. coli*. However, it is surprising that the minus strand in the pRG5-transformed strain is very poorly expressed compared with the plus strand in the pRG4-transformed strain, although involvement of the same pBR322 promoter in both cases is suspected. This might imply that the minus strand contains sequences which signal inhibition of transcription in *E. coli*.

The interesting point is that in the pRG7-transformed strain, the transcription of *URA1* is restored. This indicates that the integration of the IS element provides the promoter activity required by *URA1* for its transcription in *E. coli*. A restriction map of this IS element shows that *Eco*RI, *Bgl*II, *Pvu*II and *Bst*EII cleave within the insertion, whereas *Bam*HI and *Hind*III do not. According to this map, the size of the IS element is

Table 2 Expression of the *URA1* region in *E. coli pyrD* strains transformed by various plasmids

Strain	Plasmid	Dihydroorotate dehydrogenase activity	Amount of RNA hybridized to the plus strand of <i>URA1</i> DNA*	Amount of RNA hybridized to the minus strand of <i>URA1</i> DNA*
<i>E. coli pyrD</i>	pRG4	7.2	200×10^{-5}	2.7×10^{-5}
	pRG5	<0.01	< 10^{-5}	2.2×10^{-5}
	pRG7	7.5	217×10^{-5}	2.3×10^{-5}

E. coli pyrD cells were grown at 37 °C in minimal medium containing ampicillin ($50 \mu\text{g ml}^{-1}$) and uracil ($20 \mu\text{g ml}^{-1}$). The dihydroorotate dehydrogenase activity was assayed as previously described² using an acellular extract from a 100-ml culture of exponentially growing cells. The activity is expressed in nmol of substrate transformed per min per mg protein.

* A 10-ml culture of exponentially growing cells was labelled for 2 min with ³H-adenine ($10 \mu\text{Ci ml}^{-1}$) of specific activity 17 Ci mmol^{-1} . Crude *E. coli* extracts were obtained by sonication. Conditions for hybridization were the same as in Table 1. The amount of hybridization was expressed as the fraction of radioactivity specifically retained versus the input.

actually ~1.2 kb and not 0.9 kb as previously estimated³. Our IS element has been shown to hybridize to IS5 (1,195 base pairs)^{8,9} using the Southern procedure¹⁰ (Fig. 2).

Until now, only IS2 elements, and in one case reported recently¹¹ an IS3 element, have been observed to activate bacterial gene expression in *E. coli* (see ref. 12 for a review). IS1 and IS5 elements were also found to activate the cryptic *bgl* operon in *E. coli* K-12, but in this case the IS elements served to disrupt an operator site rather than provide a promoter sequence¹³. IS2 was also shown to increase the expression of a cloned yeast gene (*TRP5*) in *E. coli*¹⁴. This is the first time that an IS5 element has been reported to exert a transcriptional effect on the expression of a cloned yeast gene.

It is not clear whether a sequence carried by this IS5 element is in itself sufficient to bestow promoter activity. For instance, some region(s) of the IS5 element might function as part of a promoter which would also involve yeast or/and even additional upstream pBR322 sequences. Such a possibility has already been discussed by Glandsdorff *et al.*¹¹ in the case of IS2-dependent gene activation.

This work was carried out in Professor F. Lacroute's laboratory, and we thank him for his interest in our work. We also thank Drs P. Starlinger and B. Schoner for sending us various cloned IS element DNAs, and Dr J. C. Hubert and Mrs Nguyen-Juilleret for help in some experiments. G.L. was supported by a Rhône-Poulenc fellowship.

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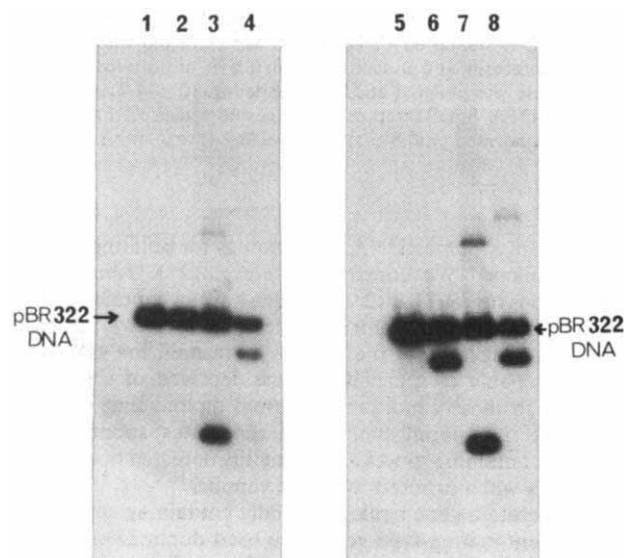


Fig. 2 Hybridization of the IS element controlling the expression of *URA1* to IS5. pDI14 is a pBR322 plasmid carrying a 3-kilobase insert of *Haemophilus haemolyticus* DNA at the *Pst*I site. pDI40 only differs from pDI14 in having an IS5 element integrated inside the *H. haemolyticus* DNA segment. The structures of these plasmids are described in ref. 9. The DNAs run on 0.8% agarose gels were as follows: lanes 1 and 5, ~4 ng of *Hind*III-digested pRG5 DNA; lanes 2 and 6, ~4 ng of *Hind*III-digested pRG7 DNA; lanes 3 and 7, ~4 ng of *Pst*I-digested pDI14 DNA; lanes 4 and 8, ~4 ng of *Pst*I-digested pDI40 DNA. After electrophoresis, the DNAs were transferred to a nitrocellulose filter¹⁰. Native pDI14 and pDI40 DNAs were made radioactive by 'nick-translation'¹⁸ (specific activity of each DNA was ~ 10^7 c.p.m. μg^{-1}). Nick-translated pDI14 DNA was hybridized to the transferred DNAs of lanes 1–4 and nick-translated pDI40 DNA was hybridized to the transferred DNAs of lanes 5–8. The *Hind*III insert of pRG5 DNA hybridizes neither to pDI14 DNA nor to pDI40 DNAs; the *Hind*III insert of pRG7 DNA does not hybridize to pDI14 DNA but does hybridize to pDI40 DNA.

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Molecular rearrangement of mating-type genes in fission yeast

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In the wild-type homothallic strain (h^{90}) of the fission yeast *Schizosaccharomyces pombe*, the mating type of a cell changes at almost every cell division¹. Genetic analysis has suggested that mating-type switching results from copy transposition of genes from one of two storage cassettes into an active site². This model is functionally similar to that already established for *Saccharomyces cerevisiae*³ but is different in that the three mating-type genes involved are tightly linked in *S. pombe*. Heterothallic strains in which mating-type switching is much reduced or absent arise from the homothallic wild type either through the irreversible loss of one of the silent storage cassettes (generating heterothallic stable minus strain h^{-S}) or through a genetic change which has been formally interpreted as a change in the switching signal at the mating-type locus (generating heterothallic normal plus (h^{+N}) and unstable minus (h^{-U}) strains). We have now isolated one of the mating-type genes *mat-P*, and have used it as a hybridization probe for sequence changes at the mating-type locus. We confirm that the genetic loss suggested for h^{-S} is due to deletion and demonstrate that the impairment of switching in h^{+N} and h^{-U} is correlated with a duplication.

The genetics of the mating-type locus are summarized in Fig. 1. To test this model at the molecular level, we have used the newly developed techniques for genetic transformation of *S. pombe*⁴ to isolate a functional copy of the *mat-P* gene from a

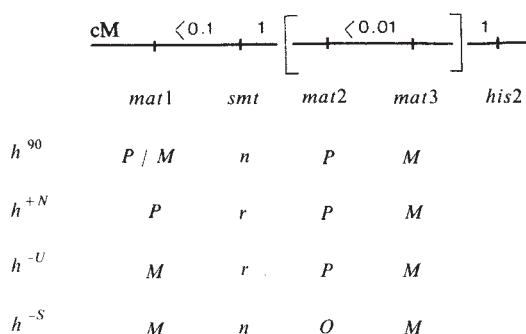


Fig. 1 Genetic map of chromosome II in the vicinity of the mating-type locus and the allocation of mating-type alleles to homothallic (h^{90}) and heterothallic h^{+N} , h^{-U} and h^{-S} strains of *S. pombe*⁵. The momentary allele present at *mat1*, *P* (plus) or *M* (minus), determines the cellular mating type and has been derived by transposition from one of the two silent storage genes, *mat2-P* or *mat3-M* during the previous event of mating-type switching. The normal switching signal *smt-n* (a *cis*-acting sequence) of the homothallic strain h^{90} can change to *smt-r*, which reduces switching frequencies from $>10^{-1}$ to 10^{-4} or lower, giving rise to the slowly interconverting heterothallic strains h^{+N} and h^{-U} . The other heterothallic strain h^{-S} is presumed to be completely stable because it has lost the silent *P* information stored in *mat2* (its switching signal is still normal and can be recovered by recombination). The *mat3* gene was referred to as '*matX*' in ref. 2. It has since been mapped to the vicinity of *mat2* (R.E., unpublished results), but the relative order of *mat2* and *mat3* has not yet been determined.

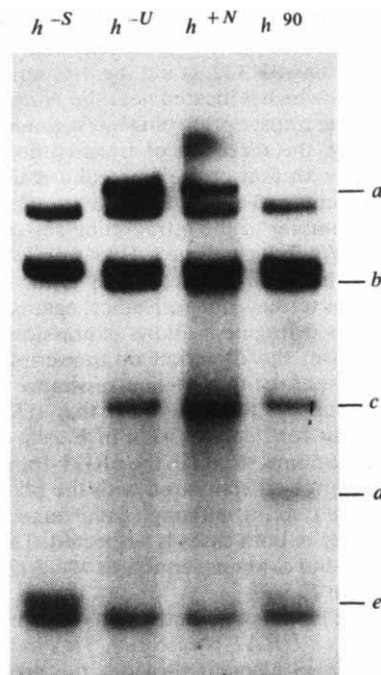


Fig. 2 Autoradiograph of ³²P-labelled pMT3.2 (nick-translated) hybridized to a Southern transfer of *Eco*RI-digested DNA of four mating-type strains, h^{-S} , h^{-U} , h^{+N} and h^{90} . All four strains are essentially isogenic. 972 h^{-S} and 975 h^{+N} were obtained originally from Urs Leupold, and h^{-U} and h^{90} were isolated as spontaneous derivatives from h^{+N} before this experiment. DNA was prepared by lysis of protoplasts⁴ in 50 mM Tris pH 7.6, 50 mM EDTA, 2% SDS at 65°C. The lysate was extracted with phenol, then chloroform, and precipitated with 0.5 vol of isopropanol. The precipitate was pelleted and resuspended in 10 mM Tris pH 7.6, 1 mM EDTA. Small samples were digested with *Eco*RI and electrophoresed on 0.8% agarose gel at 2 V cm⁻¹ for 18 h.

gene bank of *S. pombe* DNA. The strategy for isolating this gene was as follows. We constructed a diploid *S. pombe* strain homozygous for *leu1⁻his2⁻h^{-S}*; such a strain contains only the *mat-M* gene and is therefore unable to sporulate. If the *mat-P* gene is introduced into the cell via a plasmid, the cell should become capable of sporulation once deprived of a source of nitrogen. In these conditions a normal diploid that is heterozygous at the mating-type locus sporulates spontaneously. Colonies containing spores can be readily detected because they stain black when exposed to iodine vapour.

To generate a gene bank potentially containing copies of all the different mating-type genes, we used donor DNA derived from the three mating-type strains h^{90} , h^{+N} and h^{-S} . These DNAs were mixed, partially digested with *Sau*3A and ligated to the unique *Bam*H1 site in the tetracycline-resistance gene of the hybrid yeast/bacterial cloning vector pDB248 (ref. 4). pDB248 consists of the bacterial vector pBR322, the *S. cerevisiae* *leu2⁺* gene which allows selection in *leu1.32* strains of *S. pombe* and part of the 2 μm plasmid carrying the replication origin. A bank of recombinant molecules was generated in *Escherichia coli* and used to transform the diploid *S. pombe* strain, homozygous for *leu1⁻his2⁻h^{-S}*. Transformants were selected as *leu⁺* prototrophs on sorbitol-containing plates lacking leucine, at a frequency of ~1 in 2,000 viable protoplasts and 20,000 per μg of plasmid DNA. On exposure of the colonies to iodine vapour, 34 of the 70,000–80,000 transformants reacted with iodine and were found to contain spores and asci. The plasmids producing sporulating transformants were candidates for carrying an insert of *mat-P* sequences.

Three transformants were further analysed and found to be unstable. *leu1⁺* and *mat-P* were lost from ~20% of the cells

even when clones were grown under leucine selection and both genes were almost always lost together, indicating that *mat-P* is linked to *leu1*⁺ on the unstable autonomously replicating cloning vector. Spores derived from the *mat-P* transformants were germinated and 48% of the resultant colonies were found to be *leu1*⁺ and iodine positive, although the extent of staining was variable. Some clones were found to be stable for both *leu1*⁺ and *mat-P* presumably as a consequence of integration into the chromosome. To determine whether this occurs at the mating-type locus, several haploids were crossed to a *leu1*⁺ *h*⁺*his*²⁺ strain for tetrad analysis. Only one cross-over between *leu1* and *his2* was observed in a total of 81 tetrads with 4 germinating spores. As *leu1* is normally 20 centimorgans (cM) from *his2*, their close linkage of <1 cM indicates that the plasmids have integrated at the mating-type locus adjacent to *his2*. Furthermore, the *h*^{-s} haploid containing the integrated copy of the plasmid was found to have regained the mating-type switching properties of the *h*⁹⁰ strain. This suggests that integration may have occurred at *mat2*, the silent store of *mat-P* information, as integration at *mat1* would presumably result in immediate loss of *mat-P* during subsequent switching.

One of the *mat-P* plasmids was recovered from a yeast clone in which integration had not occurred, by re-transformation of *E. coli* (D.B., M. Piper & P.N., unpublished results). This plasmid, pMT3.2, was able to confer sporulating ability on diploid and haploid *h*^{-s} and on haploid *h*^{-U} strains, but not on *h*⁺*N*. It therefore carries a functional copy of *mat-P* but not *mat-M*. The plasmid pMT3.2 was digested with *EcoRI* and the lengths of the fragments generated were measured. The insert into the *BamHI* site of the vector was found to be ~10 kilobases (kb) and to contain one *EcoRI* site. As the cloning vector pDB248 has no detectable sequence homology with the *S. pombe* genome, pMT3.2 can be used directly as a hybridization probe to analyse the chromosomal organization of the mating-type locus. DNA from the four mating-type strains *h*^{-s}, *h*^{-U}, *h*⁺*N* and *h*⁹⁰ was digested with *EcoRI* and hybridized with nick-translated pMT3.2 using the Southern procedure⁶ (Fig. 2). The most striking features of the hybridization pattern are as follows. Although the *S. pombe* insert in pMT3.2 contains only one *EcoRI* site, the probe hybridizes to six bands in the *h*⁹⁰ strain, and thus some of the insert sequences must be present in more than one copy within the genome. Compared with *h*⁹⁰, the *h*^{-s} lacks three bands *b*, *c* and *d* (Fig. 2) at 5.4, 4.0 and 3.0 kb, respectively, while one small new band (Fig. 2, 1.7 kb) is apparent. On the other hand, in both *h*⁺*N* and *h*^{-U} there is a prominent extra band (*a*, 7.2 kb) and loss of one weakly hybridizing band (*d*, 3.0 kb) compared with *h*⁹⁰.

The simplest interpretation of these data is that *h*^{-s} arose from *h*⁹⁰ by loss of the silent *mat-P* cassette, thereby generating a strain which never reverts to *h*⁹⁰ and lacks certain restriction fragments. The bands which *h*^{-s} and *h*⁹⁰ have in common may reflect sequence homology between the *mat-P* and *mat-M* genes, but some hybridization may be due to sequences adjacent to the *mat-P* gene which are not directly involved in mating-type determination. *h*⁺*N* and *h*^{-U} carry a duplication of mating-type information. This may have occurred in two different ways: it could result from either a faulty transposition event in which the resident gene at *mat1* was not excised during insertion of a new gene from the silent store, or unequal crossing-over between sister chromatids at the mating-type locus. If a copy of *mat-P* at the *mat1* site recombined with the same gene at *mat2* on the sister chromatid, the products would be a cell which had a duplication of *mat-P* and the other a deletion of *mat-P*. This model predicts that the arisal of *h*⁺*N* and *h*^{-s} from *h*⁹⁰ is a reciprocal event.

The duplication in *h*⁺*N* and *h*^{-U} might be expected to reduce the rate of switching. When the duplication is removed there will be a reversion to *h*⁹⁰; this occurs at a frequency of 5×10^{-4} . The Southern blot hybridization patterns do not distinguish between *h*⁺*N* and *h*^{-U} although they presumably contain different *mat1* genes. This suggests that the region which differs between *mat1-P* and *mat1-M* is not cut by *EcoRI* and is of sufficiently

similar length as to generate bands that are indistinguishable on a gel.

Our results thus indicate the way in which heterothallic strains arise in *S. pombe*. They bear only indirectly on the mechanism of homothallic mating-type switching in this yeast but the isolation of the first hybridization probe for the mating-type locus opens the way for a more refined analysis of this process.

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The iron–oxygen bond in human oxyhaemoglobin

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Knowledge of the exact nature of the iron–oxygen bond in oxyhaemoglobin (oxyHb) is essential for the understanding of cooperative oxygen binding to haemoglobin (Hb)¹. However, X-ray studies of oxyHb have previously been hindered by the tendency of oxyHb crystals to autoxidize. Here we report the stereochemistry of the haem–oxygen complex in human oxyHb, as determined by single-crystal X-ray analysis. Bent end-on geometry of the iron–oxygen bond in haem proteins, as predicted by Pauling², was first observed in the ‘picket fence’ complex³ and has since been observed in oxymyoglobin (oxymb)^{4,5}; in oxyerythrocruorin, however, the Fe–O–O bond is almost linear⁶. In oxyHb the Fe–O–O bond angle is 156°, intermediate between the ‘picket fence’ compound and erythrocruorin. The position of N^ε of His E7 in the α -subunit suggests that it forms a hydrogen bond with the bound oxygen, as in oxymb⁷. In the β -subunit, however, N^ε of His E7 is located further from the oxygen, suggesting that the hydrogen bond is weaker.

Crystals of human oxyHb were grown according to Perutz⁸, with minor modifications to retard autoxidation. Reflections to 2.1 Å resolution were recorded with an Arndt–Wonacott oscillation camera⁹ at –2 °C. After exposure, the crystals were dissolved and spectra obtained to determine the level of methaemoglobin (MetHb). None of the crystals used contained more than 10% MetHb. Details of crystallization, data collection and processing will be published elsewhere.

The human carbomonoxyhaemoglobin (HbCO) model¹⁰ served as a starting point for refinement, using the Jack–Levitt combined X-ray and energy method¹¹. At present, the agreement between observed and calculated structure factors, expressed as a conventional *R* factor, is 0.22. Positional standard deviations of atoms were estimated using a least-squares matrix⁵ and Luzzati plot¹².

Table 1 summarizes the stereochemistry of the haem–oxygen complexes in oxyHb, oxymb and the ‘picket fence’ compounds. Figure 1 shows the striking difference between the Fe–O–O bond angles in oxyHb and oxymb, that is, 156° (±10) in both subunits of oxyHb compared with 115° (±5) in oxymb⁵. This results from differences (0.5–1.5 Å) in the location of the distal residues (that is, His E7, Val E11 and Phe CD1) in contact with the ligand. Because of a change in position of the E helix and CD

Table 1 Geometry of haem-oxygen complexes in oxyHb, oxyMb and picket fence compounds

	Fe—Ct(Å)	Fe—N(porph) (Å)*	Fe—N ^e (F8) (Å)	Fe—O1 (Å)	F8—N1FeN3(°)
OxyHb (α -subunit)	0.13 (8)	1.98 (4)	1.95 (10)	1.67 (8)	11
OxyHb (β -subunit)	-0.08 (8)	1.96 (9)	2.06 (9)	1.83 (13)	27
OxyMb (ref. 5)	0.18 (3)	1.95 (6)	2.07 (6)	1.83 (6)	1
FeO ₂ (TpivPP)(1 MeIm) (ref. 19)†	-0.03	1.98 (1)	2.07 (2)	1.75 (2)	20
FeO ₂ (TpivPP) (2 MeIm) (ref. 19)†	0.086	1.996 (4)	2.107 (4)	1.898 (7)	22

Fe—Ct, distance of Fe from mean plane of porphyrin nitrogens; positive value denotes displacement towards His F8. F8—N1FeN3, angle between imidazole plane of His F8 and line N1—Fe—N3 (vertical line across haem in Fig. 1).

* Mean distance.

† 'Picket fence' compounds. TpivPP, *meso*-tetrakis[α, α, α -(*O*-pivaloyl)amido]phenyl porphyrin; 1 MeIm, 1-methylimidazole; 2 MeIm, 2-methylimidazole.

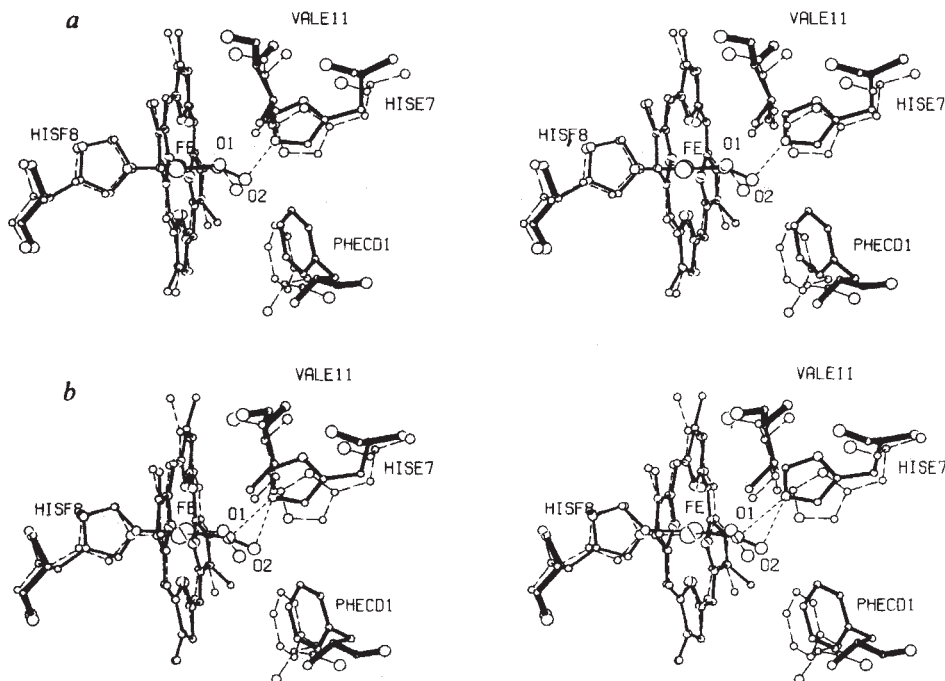


Fig. 1 Stereo view of superimposed haem environments in oxyHb subunits and oxyMb. Haems were superimposed by least-squares fit and the other residues transformed accordingly. Thick lines denote oxyHb and broken lines, oxyMb. Note the upward movement of the terminal oxygen in oxyHb, following the distal residues. *a*, α -Subunit of oxyHb and oxyMb. Dotted line indicates a probable hydrogen bond, N^e (His E7)—O₂. *b*, β -Subunit of oxyHb and oxyMb: N^e is approximately equidistant from O₁ and O₂ (dotted lines).

corner in oxyHb (see Fig. 1), these residues are further from the haem centre than in oxyMb. Thus, the haem pocket of oxyHb is more spacious than that of oxyMb. Extended Hückel calculations have shown that the iron-oxygen bond can vary between 120° and 160°, with only small consequent changes in energy (160° is, however, a favourable angle)^{13,14}. Hence, the more obtuse Fe—O—O bond angle observed in oxyHb may reflect either a reduction in steric constraints imposed upon the bound oxygen, or a different set of constraints.

Because of its loosely packed haem pocket, *R*-state Hb¹⁵ can accommodate CO, which binds to iron in a linear mode, more easily than Mb. In fact, the terminal oxygen in oxyHb (O₂ in Fig. 1) is close to the position occupied by the CO oxygen in HbCO. *R*-state Hb, therefore, has a higher O₂/CO partition coefficient than Mb, which results in reduced O₂/CO discrimination (the partition coefficient, $M = P_{1/2O_2}/P_{1/2CO}$, for *R*-state Hb is 200–250; that for Mb is 25–40)¹⁶.

Recently, Phillips and Schoenborn⁷ demonstrated by neutron diffraction that N^e of His E7 forms a hydrogen bond with the terminal oxygen in oxyMb. In the α -subunit of oxyHb, N^e—O₂ is 2.7 Å and the geometry favours a similar hydrogen bond (Fig. 1*a*). In the β -subunit, N^e of His E7 is approximately equidistant from O₂ and O₁ (3.4 Å and 3.2 Å, respectively; see Fig. 1*b*), the larger distance implying the presence of a weaker hydrogen bond with one or both oxygen atoms. Studies on the rate of formation of MetHb from oxyHb have shown that the α -subunit autoxidizes faster than the β -subunit^{17–18}. By forming a stronger

hydrogen bond, the oxygen molecule in the α -subunit may become more polarized, thus allowing more rapid autoxidation.

The detailed structure of human oxyHb will be published elsewhere.

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BOOK REVIEWS

Physicist extraordinary

Rudolf Peierls

MARK Oliphant is one of the great personalities of the world of physics, whose career has had many very different facets. Original research in nuclear physics in the Cavendish Laboratory under, and in close collaboration with, Rutherford, was followed by leadership of the radar team at Birmingham which produced the cavity magnetron and by work with Lawrence at Berkeley on the electromagnetic isotope separation for the atom bomb. After the War came the idea of the synchrotron, conceived independently and probably before others, and the creation of a nuclear physics school at Birmingham, with the construction of a cyclotron and a proton synchrotron; then, after the return to Australia, a major share in the development of the National University and, after retirement from the laboratory, a term as Governor of his native state of South Australia.

But no list of this kind could bring out Oliphant's most characteristic quality, the fearlessness with which he speaks his mind, no matter whether his thoughts are popular or unpopular, never calculating their effect on his image. While therefore what he says and writes does not always please his listeners, they always respect his intrinsic honesty. This quality came out perhaps most strongly, and created the greatest surprise, in his last, most exalted position as Governor. The men chosen for such representative positions are often senior statesmen or military men, very conscious of protocol, and maybe stuffy. Stuffiness is a quality completely lacking in Oliphant, and his direct and outspoken ways went over well in a country where stuffiness is not welcomed.

He speaks with equal directness about his failures and errors of judgement, and this has made it easier for the authors to write this biography in his lifetime. Writing about living people who can answer back, can be embarrassing, but evidently not in this case.

The authors have interviewed Oliphant and members of his family, as well as many colleagues in his different spheres, and they have had access to many letters and other documents. As a result the book brings out Oliphant's personality vividly. Perhaps the best part of the book is the account of his ten years in Cambridge. The authors do not give much detail of his papers on nuclear physics, but then they are not writing for physicists. What comes out so well is the atmosphere in the Cavendish, and Rutherford comes completely alive. Oliphant was perhaps Rutherford's

Oliphant: The Life and Times of Sir Mark Oliphant. By Stewart Cockburn and David Ellyard. Pp.369. ISBN 0-9594164-0-4. (Axiom Books, Australia: 1981.) A\$19.95.

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Oliphant in 1950, the year of his move from Birmingham to Canberra.

favourite pupil, and we understand why. The characters of these two men had much in common in their directness, and in their enthusiasm for the work. A lecture by Rutherford had made a deep impression on Oliphant when still a student, and had led to his resolve to get to Cambridge and work under Rutherford; the great respect and awe gradually gave way to genuine affection.

Yet he left Cambridge, as other collaborators of Rutherford had done, to branch out on his own in Birmingham. He took up his new post just before Rutherford's death in 1937. His intention was to build up nuclear physics in Birmingham, but the process was soon interrupted by the War. The book describes the radar work, and his abortive trip to Australia to offer his services to the Australian government when the radar problems at Birmingham had lost their urgency and the war in the Pacific was approaching his native country. Finding that no one was prepared to arrange for his services to be used, he returned to England, and soon afterwards joined E. O. Lawrence at Berkeley in work on the electromagnetic isotope separation for the atom bomb. Lawrence was another colleague to whom Oliphant had great affinity, and their relationship is also brought out very vividly here. The authors

describe the shock Oliphant felt when he heard of the use of the atom bomb on Japan, and his determination to work for solutions that would make future atomic war impossible or at least unlikely.

Back to academic life, to complete the construction of the cyclotron started before the War, and to start the proton synchrotron, based on an idea proposed by him but published by others first. The construction of two machines in a very confined space, and in a Britain beset by post-War shortages, using do-it-yourself methods as far as possible, was a typical Oliphant act of courage and optimism. The less ambitious of the machines, the cyclotron, gave little trouble; the proton synchrotron took much longer to complete than had been hoped for, and was ready only long after the similar, though somewhat larger, "cosmotron" at Brookhaven.

In fact the machine was completed only after Oliphant had left for his next assignment, the Australian National University at Canberra. The book describes the part he played in building up the spirit of the new institution, with all the struggle that involved, and also his choice of a machine for the physics department, a 10 GeV synchrotron powered by a homopolar generator. Looking back, this was clearly an error of judgement. He was again over-optimistic about the prospect and the time scale. If the machine had been completed as planned, it would have been by some years the first to reach this energy range, though even then the slow repetition rate would have made it difficult to use. As it is, only the homopolar generator was completed, but it will not drive a synchrotron and has instead found application in work on plasma physics. In spite of this disappointment, he succeeded in creating a physics department with many activities in different fields, and a staff of high quality.

Last, but not least, comes his term as Governor, which I have already mentioned. This again is described with warmth, though without glossing over some errors and embarrassments.

Taken as a whole, the book is perhaps a little longer than necessary. But it is a pleasure to read, and it does justice to a great character, whose integrity, frankness and directness set an example to us all. □

Sir Rudolf Peierls was with Oliphant in Cambridge from 1935 to 1937, and in Birmingham from 1937 to Oliphant's departure for Australia. He moved to Oxford in 1963, where he is now Emeritus Wykeham Professor.

Press Association

Membranes layer by layer

Clive Ellory

New Comprehensive Biochemistry, Vols I and II. Vol. I, *Membrane Structure*. Edited by J. B. Finean and R. H. Michell. Pp.271. ISBN 0-444-80304-1. (Elsevier/North-Holland Biomedical: 1981.) Dfl.140, \$59.50. Vol. II, *Membrane Transport*. Edited by S.L. Bonting and J.J.H.M. de Pont. Pp.362. ISBN 0-444-80307-6 (Elsevier/North-Holland Biomedical: 1981.) Dfl.140, \$59.50.

MEMBRANE expansion is a term that not only applies to certain important cellular processes, but also describes the status of the whole field of membrane biology. In addition, it seems to have become a clarion call to publishers. Predictably, however, books on the topic are extremely variable in quality, often representing unreferenced reportage at symposia. Happily, the present two works are excellent, comprising comprehensive and scholarly reviews by experts, each of whom describes the present state of knowledge in his area of membrane biology.

The depth in which subjects are covered in the first volume, *Membrane Structure*, is impressive, and is reflected by the relatively few (six) chapters of which it is composed. An initial contribution by the editors sets the scene and introduces the Singer-Nicholson fluid mosaic model — now ten years old and very much part of the established orthodoxy — as probably the last generalizable membrane model. They suggest new models will not be general, instead emphasizing the individuality of specific membranes at an advanced level of definition. Edidin produces a detailed and critical analysis of the results obtained from applying spectroscopic techniques to measuring molecular motions of membrane components, particularly the lateral diffusion of membrane proteins. op den Kamp reviews the evidence for asymmetry of both membrane proteins and lipids. Although agreeing that membrane proteins are always distributed in a completely asymmetric manner, he ends on a cautious note with regard to phospholipid asymmetry, counselling care in the interpretation of experimental results which rely on phospholipid modification during localization.

In his excellent review of membrane glycoproteins, Gahmberg starts by admitting that we do not know the reasons why all known cell surface proteins are glycoproteins. He finishes, however, on a more positive note by proposing two plausible roles for the carbohydrates attached to surface proteins.

The final two chapters deal with the demanding topics of membrane-bound enzymes (including the complexities of the adenyl cyclase system) and the structure and assembly of membrane proteins. Again the overall quality is impressive,

although some results are perhaps presented more optimistically than they deserve; for example, Freeman's discussion of Arrhenius plots could have included a word of caution on kinetic artefacts.

The credentials of the contributors to Vol. II, *Membrane Transport*, are equally impressive, but the topics are narrower or at least their treatment is less comprehensive. This is reflected numerically with 12 chapters instead of 6. Nevertheless, every contribution is worthwhile, and I particularly liked the review of anion-sensitive ATPase(s) by de Pont and Bonting, which raises the status of this system by focusing attention on the amount of information now available. In contrast, the contribution on SR Ca-ATPase seemed surprisingly lacking in data on structure and subunit composition.

The reviews on membrane permeability

for water and polar and lipophilic molecules by Sha'afi and Stein respectively, and the contribution by Weinstein *et al.* on the coupled transport of water reveal that we have made less progress towards understanding transport in membranes than we have towards elucidating their structure.

Stein's major contribution to Vol. II, on the kinetics of mediated transport, is presented with his usual rigour and vigour, and may have the consequence of promoting an uneasy conscience in those of us who publish papers defining membrane transport systems with less than the full kinetic treatment.

Technically the quality of printing and illustrations is good, and these are books which few membrane biologists can afford to be without. I hope this series can maintain its momentum and adhere to the overall title of the *New Comprehensive Biochemistry*. □

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Evolution: opinions, axioms and "isms"

Jonathan Howard

Evolution: Genesis and Revelations, with Readings from Empedocles to Wilson. By C. Leon Harris. Pp.339. Hbk ISBN 0-87395-486-6; pbk ISBN 0-87395-487-4. (State University of New York Press: 1981.) Hbk \$29.50, £20.65; pbk \$9.95, £7. *Science, Ideology, and World View: Essays in the History of Evolutionary Ideas*. By John C. Greene. Pp.202. Hbk ISBN 0-520-04217-4; pbk ISBN 0-520-04218-2. (University of California Press: 1981.) Hbk \$16.50, £11.50; pbk \$5.95, £4.

DESPITE a respectable suit of clothes, Darwinism remains deeply controversial. Even intricate internal disputes, over cladistics, over sociobiology, spill over into the public domain in extraordinary displays of bad temper in the popular press (if I may so designate, in this context, *The Times*). It is quite funny how many people feel entitled to express a public opinion regarding, say, the validity of the theory of evolution, from a standpoint of the most abysmal ignorance of any biological phenomena at all save the workings of their own bodies.

Controversy, if raucous enough, takes the reading public to the bookshops, only to be confronted with the mountain of Darwin and evolution studies. Nothing is more intellectually stultifying than to be faced with too much to read, yet more and more stuff is pressed on our attention. If, as seems likely, the evolution debate in the wide sense marks one of the most far-reaching intellectual revolutions in history, it would be a pity to devote what little time

one has to reading the wrong books. If the publishers refuse to discriminate between worthwhile and trivial books, the critics must.

Here is such a discrimination. I have two review copies of books on the history of evolutionary ideas, one a flabby paperback by a (to me) unknown biologist, the other an elegant hardback by a distinguished historian of biology. But if the general reader based his judgement on these superficial observations, and settled for the elegant book by the distinguished author, he would make a grave mistake. For it is not worth his attention, while the less favoured volume is brilliant and original, and still more exceptional in being very funny. *Evolution: Genesis and Revelations, with Readings from Empedocles to Wilson* is the punning, bathetic title of C. Leon Harris's primer in the history of evolutionary ideas. Brief readings from the evolutionary masters are sandwiched between essays by Harris in which he first introduces, and later discusses, his readings for the session. The great joy of the book is that it is deeply opinionated. There is nothing more agreeable than finding a scholar with a clear point of view, clearly stated, and supported with a will and a wit. Harris has all of this. His discursive essays cover an extraordinary range, each one a gem of concentrated opinion and argument which flows onto the page at a terrific pace. Sometimes, indeed, the pace seems too great for the constraints imposed by English grammar, but after reading a few pages I could

forgive Harris even that. The first few chapters of *Genesis* (in a most bizarre translation; could it be a Gideon Bible?) provoke a savage attack on the intellectual folly of modern creationism:

The idea of equal time for alternative theories is seductive to fair-minded people, but if creation is not scientifically supported it has no more right to equal time than the stork theory of childbirth.

Excerpts from Augustine and Aquinas bring an essay on the power of prejudice constructed around McCarthyism and *Close Encounters of the Third Kind*. Readings from Diderot, Maupertuis and Lamarck are followed by an essay on visual illusion as a metaphor for scientific creativity. Having planted his idea, Harris is happy to point to its limitations:

... visual illusions, though interesting and perhaps useful as models of creativity, are not as emotionally satisfying as the real thing. No scientist who has lost sleep because of the thrill of creating his own theory will underestimate the power of inspiration. We must go to another model to study this emotional component of creativity. One such model for the creative scientist happens to be a chimpanzee named Sultan, who one day discovered that he could connect two sticks and rake a banana into his cage. He got so excited over his creativity that he forgot to eat the banana.

It would be wrong to leave the impression of *Evolution: Genesis and Revelations* as a collection of quick-fire one-liners. Harris is funny and original in his approach, but he is also extremely thoughtful nearly all the time. Unexpectedly, however, he makes extraordinarily heavy weather of his discussion of Darwinian evolution, following readings from Darwin and Wallace. It is here, if anywhere, that one might hope that so clear and refringent a mind would throw new light, or at least make a new joke. Well, we get the new joke, in the shape of "Harris's First Law of Knowledge: *Belief in the truth of a theory is inversely proportional to the precision of the science*", but no new light. Indeed I am prepared to match Harris's trenchant style and say that I think his claim of axiomatic status for Herbert Spencer's phrase "survival of the fittest" is misleading at best, and at worst, wrong. I cannot accept that a noun phrase can ever be an axiom. 'Survival of the fittest' is no more an axiom than "C. Leon Harris". Let us suppose that Harris intends "the fittest survive" every time he says "the survival of the fittest". Does this truism indeed lie at the root of Darwinism, and is it really necessary to assimilate it as an axiom to appreciate the quality of Darwin's thought? Certainly not. The phrase, and its axiomatic form, are sloppy portmanteaux. The foundations of Darwinism are generalizations from experience and lend themselves easily to falsification. They are acceptable because they have not yet been falsified. To put the generalizations simply, there is heritable variation between

individuals, and individual reproductive performance is not independent of individual variation in other characteristics. Not as catchy as "survival of the fittest", but at least nearly right.

If "survival of the fittest" can lead so acute a mind as C. Leon Harris's into folly, we should perhaps learn to avoid the coiner of the phrase, in case he should disguise any more complex truths as simple falsehoods. I propose that books on the history of evolutionary ideas are to be avoided in direct proportion to the number of references they make to Herbert Spencer. Using this criterion, we should certainly select Harris, with not a single reference, and A.O. Lovejoy's classic *The Great Chain of Being*. By the same lights we should unhesitatingly reject John C. Greene's *Science, Ideology, and World View*, the second of my two books. Greene devotes almost as much space to Spencer as to Darwin, even coining the unhappy term Spencerianism-Darwinism to encapsulate his sense of a zeitgeist. Spencer had a total inability to make himself clear. He also wrote at inordinate length. (Publishers were more responsible in the nineteenth century: one of the most important facts about Herbert Spencer is that he was obliged to publish the ten monstrous volumes of the *Synthetic Philosophy* at his own expense). It is no surprise, therefore, that the working of Herbert Spencer's thin ore had to wait until the mother lodes had been worked out. If C. Leon Harris lives in a world of bright lights and dark shadows, John C. Greene lives in a penumbra of obscure "isms" (a horrific word which actually introduces one of his essays). In a parody of scholarship, Greene includes part of some correspondence between himself and Ernst Mayr, concerning Greene's doomed attempt to confine the use of the word "Darwinism" to a package of threadbare Victorian prejudices. As Mayr rightly says,

You may make a few sociologists happy by inflicting that package of Spencerian concepts upon Darwin, but those who probably use the word Darwinism the most will simply ignore it.

Where I have the greatest sympathy for John C. Greene's book is at the end of his last essay where he appeals with a gentle candour on behalf of the moral imagination of man against the spiritless encroachments of prescriptive sociobiology. Harris, too, ends his book with prescriptive sociobiology, but with characteristic combativeness he uses a muddy piece of Wilsonian gobbledygook as an excuse for whipping "the Marxist opponents of biological determinism". This may be another elaborate C. Leon Harris joke, but it is subtly done and for once I cannot see even a hint of a smile.

Jonathan Howard is at the ARC Institute of Animal Physiology, Babraham, Cambridge, and author of *Darwin*, recently published by Oxford University Press.

Scattering wisdom

Roger Pynn

Pulsed Neutron Scattering. By C.G. Windsor. Pp.432. UK ISBN 0-85066-195-1; US ISBN 0-470-27131-0. (Taylor & Francis/Halsted: 1981.) £25, \$85.

A GENERATION ago, a mutually beneficial relationship between solid-state physicists and the growing community of reactor specialists resulted in the birth of neutron scattering. Subsequently this field has grown to attract scientists from many other disciplines; chemists, biologists, metallurgists and even archaeologists have profited from the ever-increasing sophistication of neutron spectrometers.

Now, as reactor-based facilities are nearing their maximum performance, a new symbiotic relationship is beginning as neutron scatterers take over unfunded particle accelerators to produce pulsed neutron sources. This book describes such sources and the spectrometers which will use them. Although the book will probably be used principally for reference, the writing is sufficiently fluent that it can be read from cover to cover without undue stress. Indeed, the reader who does so will be rewarded by the occasional throw-away line incorporating practical wisdom in a pleasantly unpretentious style. The author has avoided irrelevant detail and the explanations are generally easy to follow. This is in no small measure the result of an excellent use of figures to explain the physical conformation of assorted hardware, as a guide through reciprocal space and to present illustrative experimental data. Mathematics, too, has been used intelligently. Where a mathematical derivation will be instructive, it is provided; otherwise results are usually quoted with sufficient reference to original work to allow the enthusiastic reader to find the derivations if he should need them. The simple, but usually neglected, expedient of "putting the numbers in" is frequently used to good effect, leaving the reader with a feeling for quantities as disparate as energy-resolution and moderator size.

As well as being an excellent compendium of much currently accepted wisdom, the book contains a number of contributions which are new. For example, detailed comparison of the performance of reactor and pulsed-source spectrometers has not previously reached a wide audience. Although not uncontentious, Dr Windsor's contribution in this field will certainly quell much argument — a curious effect often observed when fact is injected into a debate. The book also provides a

Academic Press have issued a condensed edition of Daniel Hillel's twin volumes, *Fundamentals* . . . and *Applications of Soil Physics* (for review see *Nature* 295, 466, 1982). The new book, *Introduction to Soil Physics*, costs \$22.50, £14.50 in paperback.

basic practical guide indispensable to anyone attempting an experiment with a pulsed-neutron spectrometer; in fact the numerous general remarks about experimental technique and spectrometer design are equally useful for the reactor-based scientist and the book should be compulsory reading matter for any new graduate student in the field. It is refreshing to find a discussion of the health hazards associated with working in weak irradiating fields; one wonders why a subject of such enormous potential importance has escaped attention in previous treatises on neutron scattering.

Apart from trivial typographical errors, I can fault the book only on two minor scores. In my view the first chapter does not do justice to its title of "Beyond Thermal Neutrons". I was expecting to be told of new science to be studied but read only the standard liturgy on the usefulness of neutron scattering. The chapter is really a thumbnail sketch of classical problems in neutron scattering and should have been titled as such. My second, less serious, objection concerns the partial use of SI units (for lengths only apparently). This is insufficient to satisfy the pedant but is more than enough to be irksome to those schooled in the conventional usage. □

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Relating to halogenated hydrocarbons

Alastair Hay

Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products. Topics in Environmental Health, Vol. 4. Edited by R.D. Kimbrough. Pp.406. ISBN 0-444-80253-3. (Elsevier/North-Holland Biomedical: 1981.) \$95, Dfl. 195.

TEN years ago the only environmental pollutant people were familiar with was DDT. Today you could list dozens of additional names. Two types of chemicals which would be included are the polychlorinated biphenyls (PCBs) and the polychlorinated dibenzodioxins (PCDDs).

Both the PCBs and the PCDDs, known collectively as chlorinated hydrocarbons, have been widely discussed, but the halogenated isomers of these compounds are not as well known. This new book on halogenated hydrocarbons will remedy this situation. Here, for the first time in a single volume, the properties of the PCBs and PCDDs are considered in relation to the whole family of halogenated hydrocarbons.

In the case of the PCBs there is some irony in the fact that the very physical properties which made these chemicals so popular are the reasons why they are so much of a problem today. The attributes of the PCBs were said to include thermal stability and resistance to both oxidation and chemical degradation. An estimated 1 million tons have been produced since 1930.

Numerous surveys have shown that the residues of PCBs can be detected in human beings. The problem appears to have been caused by eating food — and fish in particular — contaminated with PCBs, simply because there is such an abundance of these chemicals in the environment. Once these facts were known the bottom dropped out of the PCB market. Government regulations — long overdue — helped to hasten their demise. But it was only production which was affected; we still have the mess to clean up.

It is a different problem with the PCDDs, which are produced as unwanted by-products in the course of making chlorinated phenols. Often they remain as contaminants in the finished phenolic product, some of which are widely used. Pentachlorophenol, for example, is a common wood preservative. And 2,4,5-trichlorophenol — contaminated with the highly toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) — is the starting point for the manufacture of the herbicide 2,4,5-T.

Although the threat from environmental exposure to both PCBs and PCDDs is still unknown, there is much more evidence about the hazard to workers occupationally exposed to them. It emerges that, as far as toxicity is concerned, there are

many similarities between the two substances.

In her introduction to this book, Renate Kimbrough says that it was thought to be desirable to discuss PCBs, PCDDs and related compounds in one book to help convey "a better understanding of their action and the complexities of their toxic effects".

The book does just that and it does it very well. Kimbrough has persuaded leading scientists in the field to discuss the chemicals in depth and to draw comparisons between them wherever possible.

The subjects included cover everything from the chemistry of these chemicals to their production, usage, and animal and human toxicology. In addition, there are valuable case studies of human populations exposed to PCBs in Japan and to TCDD at Seveso in Italy. The last chapter, by the editor herself, discusses the problems and health effects of occupational exposure to such substances.

The book is likely to become one of the standard texts for both students and researchers concerned about the health effects of exposure to PCBs, dioxins and their ilk. It is a joy to read a book as well edited as this, where there is no overlap between chapters and where all the contributors have stuck to their brief. The reader is left in no doubt that the toxicity of these halogenated aromatic compounds is indeed complex, but that there are many similarities between such chemicals in the way they exert their toxic effects. □

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Foram stratigraphy

F.T. Banner

Stratigraphical Atlas of Fossil Foraminifera. Edited by D.G. Jenkins and J.W. Murray. Pp.310. UK ISBN 0-85312-210-5; US ISBN 0-470-27191-4. (Ellis Horwood/Halsted: 1981.) £25, \$79.95.

IN ONE concise yet comprehensive handbook, the value of fossil foraminifera to the stratigraphical geology of the British Isles is demonstrated at last. Before this publication, the information was scattered and no clear over-view could readily be obtained. Now, a collaboration by 23 authors (drawn from the petroleum industry, industrial consultancies, universities and the Institute of Geological Sciences) has provided a summary of all useful, published information, and careful



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editing has presented it systematically and intelligibly.

Each of the 12 chapters outlines the stratigraphical base for the micropalaeontology of each successive geological interval. The foraminiferal record of the Pre-Carboniferous is poor, but in succeeding strata the value of foraminifera to their biostratigraphy becomes very apparent. The seven stages which comprise the British Carboniferous succession may be recognized and divided on the distribution of foraminiferal genera alone. The following chapters on the Permian, Triassic, Jurassic, Cretaceous, Palaeogene, Neogene and Quaternary intervals demonstrate how the stages, macrofossil zones (where they exist) and principal lithostratigraphic units are characterized by foraminiferal genera or species and by their assemblages. One short chapter summarizes the available information on the North Sea Cenozoic sequences.

In all, 570 important foraminiferal taxa are illustrated (usually by scanning electron microscopy, but, where hard limestone lithofacies predominate, by optical thin sections), and for each the essential taxonomy and morphology are outlined and the source of the illustrated specimen is noted. Where it is possible to do so, stratigraphic ranges (as syntheses or as objectively recorded occurrences) are illustrated by graphs. Each chapter on stratigraphy also records the whereabouts of the more important reference collections. Where it can be attempted, there are discussions on palaeoenvironmental and palaeofacies influences on the stratigraphical record; the last short chapter outlines the successions of foraminiferal assemblages recognized through the whole Phanerozoic, as a product of both evolution and of changing environments.

This is a most useful book; it will become a standard reference for all stratigraphic geologists and micropalaeontologists concerned with the post-Devonian strata of the British Isles. The format is clear, convenient for reference and methodical; the printing of text, diagrams and photographs is to a high standard. Some users will argue with the nomenclature which has been employed, and others will fault the taxonomy, but this is inevitable. More serious criticisms are that the essentially provincial scope of the book is not indicated by its title, and, in some chapters more than others, the welter of local British place-names (not adequately clarified by location maps) will confuse the overseas reader. This said, the editors, authors, publishers and the British Micropalaeontological Society are to be congratulated on this concise, attractive and much-needed volume. □

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Rules for the environmental game

David Dickson

DDT: Scientists, Citizens and Public Policy. By Thomas R. Dunlap. Pp.318. ISBN 0-691-04680-8. (Princeton University Press: 1981.) \$24.50, £13.10. *The Reserve Mining Controversy: Science, Technology, and Environmental Quality.* By Robert V. Bartlett. Pp.293. ISBN 0-253-14556-2. (Indiana University Press: 1981.) \$17.50, £10.50.

"I've never seen a company, even among the most enlightened, that considered environmental affairs to be anything more than a public relations problem" one of the three members of President Carter's Council on Environmental Quality said shortly after returning to his previous role as an environmental activist earlier this year.

The charge is far from one-sided. In an important sense, the various environmental debates that have taken place in the United States over the past 20 years have, indeed, been part of a large public relations game. Refusing to believe that a serious problem exists, many private companies have been more concerned with demonstrating the legitimacy of their existing practices than with volunteering the need for stronger federal restrictions. For its own part, the environmental movement recognized at an early stage the need to operate in a highly-visible public arena. Close relations with the mass media, reinforced by a well-developed sense of what makes a good news story, helped generate a mostly-sympathetic press, which in its turn served to mobilize public support.

Behind the public relations front, of course, more serious issues have been fought out. The growth of the environmental movement in the early 1960s was one of the first significant challenges to traditional mechanisms for the control of both industrial technology and its side-effects. Previously, discussion about the environmental impact of new industrial or agricultural practices was contained largely within professional circles of scientists and physicians. The public had little cause — or inclination — to question the views of those it accepted as experts. The environmental movement openly challenged this mould. What started as a few isolated protests soon snowballed, in the face of apparent official indifference, into a nation-wide bandwagon on which many politicians were quick to jump. In the process, channels for public participation were opened up that significantly increased the opportunities for non-professional groups to intervene in the process of determining what technical innovations were and were not socially desirable. The most important development was the National Environmental Protection Act of 1969. By permitting environmentalist

groups to question the scope and adequacy of "impact statements" required for any major federally-funded project, NEPA provided a platform from which challenges to a wide range of government initiatives could be launched.

Even before NEPA, however, the environmentalist movement had found that, if public relations was its most effective political weapon, the courts were its most advantageous battle-ground. The courts were relatively neutral territory where environmentalists could set their own agenda and argue on equal terms with government administrators and industrial manufacturers alike. No where were the benefits of this strategy more clearly demonstrated than in the campaign which eventually led to the decision of the Environmental Protection Agency to ban the use of DDT in 1972.

Production of DDT in the United States had risen to about three million pounds a month by the end of the War, and shortly afterwards it entered civilian use with a reputation of being such a miracle chemical that one entomologist speculated that it might eventually banish all insect-borne diseases from the Earth. Yet as its use to protect agricultural crops, promoted by government scientists, grew rapidly, the first reports of mortality among birds and fishes began to be heard from wildlife biologists, indicating that DDT might not be the complete miracle that it appeared.

The early reports were not ignored. But neither, as Thomas Dunlap demonstrates in his closely-researched history of the campaign against the pesticide, *DDT: Scientists, Citizens and Public Policy*, were they initially taken very seriously. To farmers and pesticide manufacturers, the death of a few robins was a small price to pay for the benefits that the pesticide had brought.

The turning-point came with the publication of Rachel Carson's *Silent Spring* in 1962. Mrs Carson presented the various data that biologists had collected showing the different effects on wildlife that DDT seemed to be having, in particular how it was being concentrated through the food chain. But she went further, using the raw data to build an indictment of modern industry that soon became a battle-cry for the environmentalist movement, turning many conservationists into political activists.

If it took only one individual to open up the issue, more effort was needed to secure political change. Much of Dunlap's book is taken up with a close description of the case against DDT that was presented in 1968 by the newly-formed Environmental Defense Fund against the state of Wisconsin. Selecting its legal ground carefully, the EDF was granted a legal hearing to evaluate its claim that the scientific

evidence against DDT, for instance its link with declining bird populations such as that of the peregrine falcon, was sufficient to have the chemical declared an environmental pollutant for the purposes of regulation.

The EDF won its case. A sympathetic judge agreed that the evidence against DDT was sufficiently damning, and that there was no identifiable level beneath which DDT could be claimed to be completely harmless, a conclusion which undoubtedly contributed to the EPA's decision to phase out the chemical in 1972. Yet as much as the verdict itself, it was the publicity generated by the court case that provided some of the most effective spin-off. For it helped to generate a wave of public support for strict environmental laws — such as NEPA — that sympathetic legislators were quick to pass through Congress.

Industry, too, learnt its lesson from the Wisconsin hearing. The National Agricultural Chemicals Association initially sent only one retired attorney, a former counsel for the Velsicol Chemical Corporation, to present its defence of DDT. Having little reason to doubt that the arguments about the lack of evidence of DDT's harmfulness, which had proved successful in previous government regulatory proceedings, would again prevail, he was unprepared for either the style or the strength of the environmentalists' carefully-prepared attack. Even if it was a public relations battle, the symbolism of the loss in Wisconsin had to be taken seriously — a message which the chemical industry gradually took to heart during the 1970s.

The path seen in the struggle against DDT, culminating in hearings before the Environmental Protection Agency in 1971 and its eventual ban by EPA administrator William D. Ruckelshaus, was soon to become familiar. A similar process led to the ultimately-successful efforts by residents of Wisconsin's neighbouring state, Minnesota, to stop the Reserve Mining Company from discharging waste tailings from its taconite mill directly into Lake Superior, the largest inland sea in the Western Hemisphere.

In *The Reserve Mining Controversy: Science, Technology and Environmental Quality*, Robert V. Bartlett describes how the final outcome of that campaign, fought in various state and federal courts over a period of more than ten years, was as much the result of changing public perceptions as a neutral assessment of scientific evidence that the tailings discharged into the lake were causing harm.

The parallels with the DDT campaign are close. Just as the first critics of DDT based their case on the environmental effects of the pesticide which much of public opinion could dismiss as relatively harmless, so the first complaints against Reserve Mining, which had been given permission to discharge its tailings into the

lake when building its plant in 1947, focused on the discoloration of the water that the discharge appeared to cause, even though there was no direct evidence that the discoloration was causing significant biological damage.

Similarly, just as the final nail in DDT's coffin was the discovery early in 1969 that the chemical was a potential human carcinogen, so the federal order requiring Reserve Mining to find a land-based depository for its tailings — the result of a drawn-out suit against the company filed by the Department of Justice at the instigation of the EPA — hinged on the close morphological similarity between particles in the taconite tailings and asbestos fibres which scientists were beginning to demonstrate posed a significant hazard to health.

Bartlett, like Dunlap, provides a useful account of the twists and turns of the legal proceedings. The Reserve Mining controversy provides, as he points out, perhaps one of the most dramatic examples of the difficulties facing courts that are asked to decide on relatively complex scientific issues with legal equipment often ill-suited to the task. Each side in the dispute exploited the ambiguity of evidence about the environmental and health effects of the taconite waste to its own advantage, applying criteria of "truth" and "legitimacy" entirely foreign to conventional scientific discourse. As Bartlett points out, the dilemmas faced by the courts on such issues are partly the result of built-in tendencies of the political system in the United States to try to resolve conflict through adversary litigation rather than consensus-based planning. Dunlap, however, goes further to argue that the environmental problems raised by persistent pesticides such as DDT are not merely the result of unthinking industrial growth, or shifting public perceptions, but have deeper social and political roots. Among these, he suggests, is a "technical-fix" mentality to pest control that was reinforced by the professional self-image of economic entomologists, physicians' concepts of health and disease, and the economic motivations of farmers and pesticide manufacturers.

The significance of this message is unlikely to disappear. The climate of public opinion has changed since the days when the environmentalist movement was at its height, yet the change may not be as dramatic as many in the Reagan administration would like to believe. Plans to overhaul the Clean Air Act, for example, offered as a pre-election promise to the auto industry in 1980, have been postponed largely on the grounds that the political price in terms of lost popular support would be too high. Rather than abandoning the ideal of environmental protection, the administration is seeking more subtly to change the ground-rules.

The lesson of both of these books is the need to treat semantics warily. In both the DDT and the Reserve Mining disputes, the

struggle over the proper treatment of the natural environment became clothed, for legal purposes, in a scientific language that as often seemed to obscure as clarify the real nature of the conflict. The problem is not unique to the United States, but it tends to be exacerbated in a country where, as one social scientist recently put it, "the gap between political ideal and political reality is a continuing central phenomenon". □

David Dickson was Washington News Editor for Nature from 1977 to 1982.

AI in chemistry

R. Venkataraghavan

Applications of Artificial Intelligence for Organic Chemistry: The Dendral Project. By Robert K. Lindsay *et al.* Pp.194. ISBN 0-07-037895-9. (McGraw-Hill: 1981.) \$39.50, £19.95.

Applications of Artificial Intelligence for Organic Chemistry provides an excellent overview of a discipline that is playing an increasingly important role in problem-solving techniques using computers. The volume takes the reader through the definition of a problem in organic chemistry, concepts of advanced computer techniques, applications and, finally, to some interesting observations and conclusions of the authors. The applications cited in the book represent the research carried out by a team of interdisciplinary scientists, and provide some unusual and thought-provoking perspectives on the promise of computing in this area.

Emphasis is placed on the utilization of spectrochemical data and Dendritic Algorithms for structure-elucidation problems. The evolution of the Dendral project and its current capabilities clearly show the potential of computer-based techniques in the symbolic manipulation of complex information. The application of these techniques, however, is not confined to organic chemistry; in time they will undoubtedly be used to tackle a number of other problems in applied science. Certainly, the discussions of design considerations and the choice of computer-based tools should benefit those readers involved in the development of a variety of complex software projects.

This concise volume, representing the fruits of several years of active research, will prove to be a valuable source book for all seriously interested in the applications of computer techniques to problems in organic chemistry. It is to be hoped, too, that its influence will spread to associated disciplines and beyond. □

R. Venkataraghavan is the Manager of the Research Applications Development Department at Lederle Laboratories, Pearl River, New York.

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